REMARKS

Claims 1-14 were previously pending in this application. In response to the Election/Restriction Requirement in the Office Action mailed April 17, 2008, Applicant elects claims 1-8 of Group I with traverse. Applicant further elects the species "a subject at risk of developing" with traverse for examination. Applicant also elects species "obesity" with traverse for examination. Applicant elects the species *Nitrosonomas* with traverse for examination.

Claims 9-14 are withdrawn from consideration without prejudice or disclaimer. No new claims have been added. As a result, claims 1-8 are pending for examination with claim 1 being independent. No new matter has been added.

ELECTION/RESTRICTION

Originally filed claims 1-14 were restricted into three groups of inventions: Group I including claims 1-8, drawn to a method comprising positioning ammonia oxidizing bacteria in close proximity to a subject for treating a subject with or at risk of developing any numerous conditions; Group II, including claim 9, drawn to a method of augmenting animal growth by removing ammonia oxidizing bacteria from the surface of the animal; and Group III, including claims 10-14, drawn to a method of using ammonia oxidizing bacteria in the manufacture of a medicament for providing nitric oxide to a subject who is at risk of developing or has developed any of a number of conditions.

Applicant respectfully traverses the restriction requirement because no undue burden would result in conducting a search and examination of all the originally filed claims. That is, a search directed to the ammonia oxidizing bacteria production of nitric oxide of the present invention would uncover relevant art pertinent to performing an examination of the claims directed to effects on health in subjects including humans an animals and a corresponding medicament and method of treatment . In addition, the M.P.E.P is quite clear in stating that for a restriction requirement to be proper, the inventions must be independent and distinct. See M.P.E.P. §802.1. The term "independent" means "there is no disclosed relationship between the two or more inventions claimed." Id. In contrast to the definition described in the M.P.E.P. there is a disclosed relationship between the two or more claims groupings- namely, the use of ammonia oxidizing bacteria to produce nitric oxide to provide health benefits to a subject.

Therefore, Applicants respectfully request reconsideration and withdrawal of the election/restriction requirement and examination of all the originally filed claims.

Species

Applicant disagrees that independent claim 1 and dependent claim 2 are not generic and that the species are not art recognized equivalents. Specifically, Applicant disagrees that "a subject who has developed a condition is not an art-recognized equivalent for one who is merely at risk of developing the same at some point in the future." All conditions exist on a continuum. Precisely where on that continuum the symptoms reach clinical significance and so meet the clinical definition of that condition to warrant treatment is arbitrary as is known by physicians skilled in the art of diagnosing and treating patients for chronic conditions. Thus in addition to diagnosis after onset of symptoms, an important part of treatment includes preventative medicine. The sooner effective treatment can be initiated, the more effective that treatment is so that more serious complications can often be avoided.

As is known, the clinical decision to treat any condition depends on the severity of the symptoms, the degree of dysfunction those symptoms are causing, the clinical course of the condition, and the side effects of any treatment. The side effects of any treatment are balanced against the effects of the untreated condition. As new treatments are developed with fewer side effects, treatment is initiated earlier in the course of the disease. As Evidence Based Medicine develops a better understanding of the course of the disease and which treatments affect which symptoms and with what side effects, the recommended course of treatment changes.

It is generally recognized by those skilled in the art as well as the general public that preventing disease is more effective than treating it after onset of symptoms. Current treatment guidelines for heart disease particularly address the prevention of heart disease rather that treatment after it has occurred.[1] In fact, the major medical focus in treating heart disease is prevention through the identification of risk factors which increase the risk that an individual will develop heart disease. The treatments administered by physicians for individuals who have heart disease (i.e. who have suffered a heart attack) and for individuals who are merely at risk for

[1] *Guidelines on Diabetes, Pre-Diabetes, and Cardiovascular Diseases*, European Heart Journal Supplements, 9, Supplement C, C3-C74 (2007) is included in Appendix A.

developing heart disease are the same, that is, the control of blood pressure, blood lipids, weight, exercise, stress reduction, and so on.

Moreover, many diseases are cryptic in that their presence or absence is to some extent ambiguous so that there is no clear line delineating a difference between a person who has a disease and a person who is merely at risk for the disease. When a risk factor such as hypertension is treated, the symptom goes away so that a person undergoing treatment for hypertension with normalized blood pressure no longer has hypertension, although the person remains at risk for hypertension. The treatment for hypertension therefore continues on the basis of the risk that the person would acquire hypertension were the medication stopped. Correcting a disorder before it reaches clinical significance is the ultimate goal of preventative medicine and is especially important in disorders that are developmental in nature. For example autism spectrum disorders exist on a continuum (the autism spectrum) which is primarily associated with difficulties in communicating and socialization. Difficulties in socialization early on cause worse prognosis later because social skills that are not developed early impede acquisition of different social skills later. There is a cumulative effect that is known to be best addressed as early as possible. As such a subject who has developed a condition and a subject at risk for developing that condition are not independent or distinct species. However, Applicant elects "at risk of developing" as a species for examination purposes only.

Similarly, the diseases and disorders treatable with aspects of the present invention are all characterized by low nitric oxide as a fundamental aspect of the disease. Many sequelae of those diseases occur only after a long time. Raising the NO level may reduce the progression of those diseases and the sooner the NO level is raised the longer the period of time the progression will be reduced. Many of these diseases are associated because they share the common physiological pathway of low nitric oxide. For example, Appendix A provides a discussion of how NO is involved in vascular diseases.[2] Increasing the nitric oxide level according to aspects of the present invention may improve disorders that have reached clinical significance as well as disorders that have not yet reached clinical significance. As such, the diseases recited in claim 1 share a common characteristic of low nitric oxide and are not independent and distinct species. However, Applicant elects obesity as a species for examination purposes only.

Finally, Applicant disagrees that the bacteria recited in dependent claim 2 are mutually exclusive. One aspect of the invention is the use of autotrophic ammonia oxidizing bacteria. These bacteria represent a single class of bacteria, all related to each other by virtue of their unique property of having the protein ammonia monooxygenase which catalyzed the energy linked oxidation of ammonia to nitrite. As was known in the art at the time of invention, all bacteria in this class have this enzyme[3] but no bacteria outside of this class have it. Appendix B illustrates that these two different ammonia oxidizing bacteria (*Nitrosomonas Europea* and *Nitrosococcus*[4]) have ammonia monooxygenase enzymes that are extremely similar in all chemical properties.

Validation that ammonia oxidizing bacteria have similar ammonia oxidizing enzymes occurred with subsequent genetic sequencing of the AMO gene. For example, Calvó[5] subsequently confirmed that all ammonia oxidizing bacteria share the gene for ammonia monooxygenase with significant homology, and so share a common ancestor. As such, Calvó confirmed what was known in the art, namely, that ammonia oxidizing bacteria constitute a single class recognized in the art as being equivalent in respect to having the same enzyme for ammonia monooxygenase that turns ammonia into NO and nitrite. Therefore, the bacteria listed in dependent claim 2 are not mutually exclusive, nor independent or distinct species. However, Applicant elects *Nitrosomonas* as a species for examination purposes only.

Independent claim 1 as well as dependent claims 2-8 read upon all the above-elected species.

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[2] Id. at page C19.

[3] Hooper, A. and Nason, A., *Characterization of Hydroxylamine-Cytochrome c Reductase from the Chemoautotrophs Nitrosomonas europaea and Nitrosocystis oceanus*, Journal of Biological Chemistry, Vol. 240, No.10, 4044-4057 (1965) is included in Appendix B.

[4] The name for Nitrosocystis was changed to Nitrosococcus.

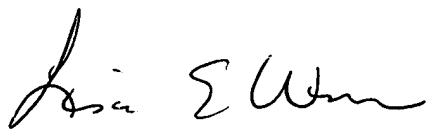
[5] Calvó L, Cortey M, García-Marin JL, Garcia-Gil LJ., *Polygenic analysis of ammonia-oxidizing bacteria using 16S rDNA, amoA, and amoB genes*, Int Microbiol., Jun; 8(2):103-10 (2005) is included in Appendix C.

CONCLUSION

In view of the foregoing amendments and remarks, reconsideration is respectfully requested. This application should now be in condition for allowance; a notice to this effect is respectfully requested. If the Examiner believes, after this amendment, that the application is not in condition for allowance, the Examiner is requested to call the Applicant's attorney at the telephone number listed below.

If this response is not considered timely filed and if a request for an extension of time is otherwise absent, Applicant hereby requests any necessary extension of time. If there is a fee occasioned by this response, including an extension fee, please charge any deficiency to Deposit Account No. 50/2762.

Respectfully submitted,
David R. Whitlock, Applicant

By: 

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Docket No.: D0460-7010US
Date: October 17, 2008

APPENDIX A

European Heart Journal Supplements (2007) 9 (Supplement C), C3–C74
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Guidelines on diabetes, pre-diabetes, and cardiovascular diseases: full text‡

The Task Force on Diabetes and Cardiovascular Diseases of the European Society of Cardiology (ESC) and of the European Association for the Study of Diabetes (EASD)

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EBAC

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‡ This is the full text version of Eur Heart J doi:10.1093/eurheartj/ehl260.

Preamble

Guidelines and Expert Consensus documents aim to present management and recommendations based on all of the relevant evidence on a particular subject in order to help physicians to select the best possible management strategies for the individual patient, suffering from a specific condition, taking into account not only the impact on outcome, but also the risk–benefit ratio of a particular diagnostic or therapeutic procedure. Numerous studies have demonstrated that patient outcomes improve when evidence-based guideline recommendations are applied in clinical practice.

A great number of Guidelines and Expert Consensus Documents have been issued in recent years by the European Society of Cardiology (ESC) and also by other organizations or related societies. The profusion of documents can question the authority and credibility of guidelines, particularly if discrepancies appear between different documents on the same issue leading to confusion for practising physicians. In order to avoid these pitfalls, the ESC and other organizations have issued recommendations for formulating and issuing Guidelines and Expert Consensus Documents. The ESC recommendations for guidelines production can be found on the ESC website. It is beyond the scope of this preamble to recall all, but the basic rules.

In brief, the ESC appoints experts in the field to carry out a comprehensive review of the literature, with a view to making a critical evaluation of the use of diagnostic and therapeutic procedures, and assessing the risk–benefit ratio of the therapies recommended for management and/or prevention of a given condition. Estimates of expected health outcomes are included, where data exists. The strength of evidence for or against particular procedures or treatments is weighed, according to predefined scales for grading recommendations and levels of evidence, as outlined below.

The Task Force members of the writing panels, as well as the document reviewers, are asked to provide disclosure statements of all relationships they may have, which might be perceived as real or potential conflicts of interest. These disclosure forms are kept on file at the European Heart House, headquarters of the ESC and can be made available by written request to the ESC President. Any changes in conflict of interest that arise during the writing period must be notified to the ESC.

Guidelines and recommendations are presented in formats that are easy to interpret. They should help physicians to make clinical decisions in their daily routine practice, by describing the range of generally acceptable approaches to diagnosis and treatment. However, the ultimate judgement regarding the care of an individual patient must be made by the physician-in-charge of his/her care.

The *ESC Committee for Practice Guidelines* (*CPG*) supervises and coordinates the preparation of new *Guidelines* and *Expert Consensus Documents* produced by Task Forces, expert groups or consensus panels. The Committee is also responsible for the endorsement of these Guidelines and Expert Consensus Documents or statements.

Once the document has been finalized and approved by all the experts involved in the Task Force, it is submitted to outside specialists for review. In some cases, the document can be presented to a panel of key opinion leaders in Europe on the relevant condition, for discussion and critical review. If necessary, the document is revised once more and finally approved by the CPG and selected members of the Board of the ESC and subsequently published.

After publication, dissemination of the message is of paramount importance. Publication of executive summaries and the production of pocket-sized and PDA-downloadable versions of the recommendations are helpful. However, surveys have shown that the intended end-users are often not aware of the existence of guidelines, or simply do not put them into practice. Implementation programmes are thus necessary and form an important component of the dissemination of knowledge. Meetings are organized by the ESC, and directed towards its member National Societies and key opinion leaders in Europe. Implementation meetings can also be undertaken at a national level, once the guidelines have been endorsed by the ESC member societies, and translated into the local language, when necessary.

All in all, the task of writing Guidelines or Expert Consensus Documents covers not only the integration of the most recent research, but also the creation of educational tools, and implementation programmes for the recommendations. The loop between clinical research, writing of guidelines, and implementing them into clinical practice can then only be completed if surveys and registries are organized to verify that actual clinical practice is in keeping with what is recommended in the guidelines. Such surveys and registries also make it possible to check the impact of strict implementation of the guidelines on patient outcome.

Classes of Recommendations:

Class I	Evidence and/or general agreement that a given diagnostic procedure/treatment is beneficial, useful, and effective
Class II	Conflicting evidence and/or a divergence of opinion about the usefulness/efficacy of the treatment or procedure
Class IIa	Weight of evidence/opinion is in favour of usefulness/efficacy
Class IIb	Usefulness/efficacy is less well established by evidence/opinion
Class III	Evidence or general agreement that the treatment or procedure is not useful/effective and in some cases may be harmful

Levels of Evidence:

Level of Evidence A	Data derived from multiple randomized clinical trials or meta-analyses
Level of Evidence B	Data derived from a single randomized clinical trial or large non-randomized studies
Level of Evidence C	Consensus of opinion of the experts and/or small studies, retrospective studies, registries

Recommendations for ESC Guidelines Production at www.escardio.org.

Introduction

Diabetes and cardiovascular diseases (CVD) often appear as the two sides of a coin: on one side, diabetes mellitus (DM) has been rated as an equivalent of coronary heart disease (CHD), and conversely, many patients with established CHD

suffer from diabetes or its pre-states. Thus, it is high time that diabetologists and cardiologists join forces together to improve the quality management in diagnosis and care for the millions of patients who have both cardiovascular and metabolic diseases in common in one and the same person. The cardio-diabetologic approach not only is of utmost importance for the sake of those patients, but also instrumental for further progress in the fields of cardiology and diabetology.

The European Society of Cardiology (ESC) and the European Association for the Study of Diabetes (EASD) have accepted this challenge and decided to develop joint, evidence-based guidelines for 'Diabetes and Cardiovascular Diseases'. Experts from both sides were asked to form a Task Force and to write state-of-the-art chapters. Although individual authors have been assigned to draft the manuscripts according to their specific area of expertise, the guidelines were then extracted and harmonized as a true team effort by the whole group. Hence, the names of all authors appear only on the cover of these guidelines as members of the writing group. Some of the members of the Task Force were helped in the literature search and writing process by members of their respective teams and these contributors are also named on the cover as contributors. The guidelines were then reviewed by independent referees appointed by the two scientific organizations whose identity were disclosed, once all criticisms and suggestions had been incorporated into the text to achieve the broadest possible expertise and consensus. The referees are also acknowledged with their names on the cover and are an important, integral part of this scientific guideline exercise.

It may seem that these guidelines are rather extensive. They were, however, written for two 'worlds', diabetology and cardiology. Thus, information that may seem obvious, including pathophysiology, for one part may need a more extensive description for the other. A decision was therefore taken, to keep the main document as complete as possible, making an executive summary and pocket guidelines for those, who are searching short, practical information. These guidelines do not aim to provide detailed information on daily blood glucose management in patients because therapies are tailored to individual patient requirements, particularly in patients with type 2 diabetes. Achieving the agreed glucose level targets is more important than the therapy and regimen. For those requiring additional information on blood glucose management the Global Guideline for Type 2 Diabetes of the International Diabetes Federation (www.idf.org) is recommended.

The core approach of the group is depicted in *Figure 1*. An algorithm has been developed to help discover the alternate CVD in patients with diabetes, and vice versa, the metabolic diseases in patients with CHD, setting the basis for appropriate joint therapy. This algorithm has also been endorsed by the expert working group of the Declaration of Vienna on February 15, 2006 under the auspices of the Austrian Presidency of the European Union. The purpose of these guidelines is to improve the management of:

(1) Patients with overt diabetes.
(2) Patients at risk of developing diabetes, as demonstrated by impaired glucose tolerance.
(3) Cardiovascular diseases in these patient populations.

The terms 'primary prevention' and 'secondary prevention' may not be quite appropriate in the case of diabetes, a high-risk situation in itself, but the terms are strongly consolidated and kept in this context when reasonable.

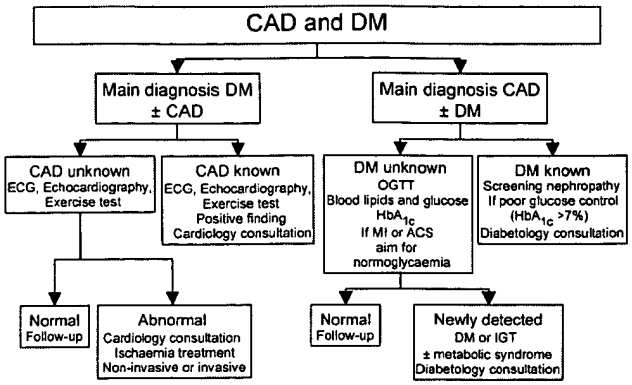


Figure 1 Investigational algorithm for patients with coronary artery disease and diabetes mellitus.

It is a great privilege for the two co-chairmen of this task force of having been able to work with the finest and best reputed experts and scientists in the field at the European level and to give these guidelines now to the community of cardiologists and diabetologists. On this occasion, we wish to thank all members of the task force who so generously shared their knowledge, as well as the referees for their tremendous input. Special thanks go to Professor Carl Erik Mogensen for his advice on the diabetic renal disease and microalbuminuria sections. We would also like to thank the ESC and the EASD for making these guidelines possible. Finally, we want to express our appreciation of the guideline team at the Heart House, especially Veronica Dean, for their extremely helpful support.

Stockholm and Munich, September 2006
Professor Lars Ryden, Past-President ESC
Professor Eberhard Standl, Vice-President EASD

Definition, classification, and screening of diabetes and pre-diabetic glucose abnormalities

Table of Recommendations:

Recommendation	Class[a]	Level[b]
The definition and diagnostic classification of diabetes and its pre-states should be based on the level of the subsequent risk of cardiovascular complications	I	B
Early stages of hyperglycaemia and asymptomatic type 2 diabetes are best diagnosed by an oral glucose tolerance test (OGTT) that gives both fasting and two-hour post-load glucose values	I	B
Primary screening for the potential type 2 diabetes can be done most efficiently using a non-invasive risk score, combined with a diagnostic oral glucose tolerance testing in people with high score values	I	A

[a]Class of recommendation.
[b]Level of evidence.

Introduction

DM is a metabolic disorder of multiple aetiology characterized by chronic hyperglycaemia with disturbances of carbohydrate, fat, and protein metabolism resulting from defects of insulin secretion, insulin action, or a combination of both.[1] In type 1 diabetes, it is due to a virtually complete lack of endogenous pancreatic insulin production, whereas in type 2 diabetes, the rising blood glucose results from a combination of genetic predisposition, unhealthy diet, physical inactivity, and increasing weight with a central distribution resulting in complex pathophysiological processes. Traditionally, diagnosis of diabetes was based on symptoms due to hyperglycaemia, but during the last decades much emphasis has been placed on the need to identify diabetes and other forms of glucose abnormalities in asymptomatic subjects. DM is associated with development of specific long-term organ damage (diabetes complications) including retinopathy with potential blindness, nephropathy with a risk of progression to renal failure, neuropathy with risk for foot ulcers, amputation, and Charcot joints and autonomic dysfunction such as sexual impairment. Patients with diabetes are at a particularly high risk for cardiovascular, cerebrovascular, and peripheral artery disease.

Definition and classification of diabetes

Since the first unified classification of diabetes by the National Diabetes Data Group in 1979[2] and the World Health Organisation (WHO) in 1980,[3] a few modifications have been introduced by the WHO[4,5] and the American Diabetes Association (ADA),[6,7] (*Table 1*).

Impaired glucose tolerance (IGT) can be recognized by the results of OGTT only: 2-h post-load plasma glucose (2hPG) ≥7.8 and <11.1 mmol/L (≥140 and <200 mg/dL).

A standardized OGTT test performed in the morning, after an overnight fast (8–14 h); one blood sample should be taken before and one 120 min after intake of 75 g glucose dissolved in 250–300 mL water in a course of 5 min (note: timing of the test is from the beginning of the drink).

Classification of diabetes includes both aetiological types and different clinical stages of hyperglycaemia as suggested by Kuzuya and Matsuda.[8] Four main aetiological categories of diabetes have been identified as diabetes type 1, type 2, other specific types, and gestational diabetes, as detailed in the WHO document[4] (*Tables 2* and *3*, *Figure 2*).

Type 1 diabetes characterized by deficiency of insulin due to destructive lesions of pancreatic β-cells; usually progresses to the stage of absolute insulin deficiency. Typically, it occurs in young subjects with acute-onset with typical symptoms of diabetes together with weight loss and propensity to ketosis, but type 1 diabetes may occur at any age,[9] sometimes with slow progression. People who have antibodies to pancreatic β-cells such as glutamic-acid-decarboxylase (GAD), are likely to develop either typical acute-onset or slow-progressive insulin-dependent diabetes.[10,11] Today antibodies to pancreatic β-cells are considered as a marker of type 1 diabetes, although such antibodies are not detectable in all patients.

Type 2 diabetes is caused by a combination of decreased insulin secretion and decreased insulin sensitivity. Typically, the early stage of type 2 diabetes is characterized by insulin resistance and decreased ability for insulin secretion causing excessive post-prandial hyperglycaemia. This is followed by a gradually deteriorating first-phase insulin response to increased blood glucose concentrations.[12] Type 2 diabetes, comprising over 90% of adults with diabetes, typically develops after middle age. The patients are often obese or have been obese in the past and have typically been physically inactive. Ketoacidosis is uncommon, but may occur in the presence of severe infection or severe stress.

Gestational diabetes constitutes any glucose perturbation that develops during pregnancy and disappears after delivery. Long-term follow-up studies, recently reviewed by Kim *et al.*,[13] reveal that most, but not all, women with gestational diabetes do progress to diabetes after pregnancy. Long-term studies that have been conducted over a period of more than 10 years reveal a stable long-term risk of ~70%.[13] In some cases, type 1 diabetes may be detected during pregnancy.

Other types include: (i) diabetes related to specific single genetic mutations that may lead to rare forms of diabetes, as for instance MODY; (ii) diabetes secondary to other pathological conditions or diseases (as a result of pancreatitis, trauma, or surgery of pancreas); (iii) drug or chemically induced diabetes.

The clinical classification also comprises different stages of hyperglycaemia, reflecting the natural history of absolute or relative insulin deficiency progressing from normoglycaemia to diabetes. It is not uncommon that a non-diabetic

Table 1 Criteria used for glucometabolic classification according to the WHO (1999), ADA (1997) and (2003)

Glucometabolic category	Source	Classification criteria mmol/L (mg/dL)
Normal glucose regulation (NGR)	WHO	FPG < 6.1 (110) + 2-h PG <7.8 (140)
	ADA (1997)	FPG < 6.1 (110)
	ADA (2003)	FPG < 5.6 (100)
Impaired fasting glucose	WHO	FPG ≥ 6.1 (110) and <7.0 (126) + 2-h PG < 7.8 (140)
	ADA (1997)	FPG ≥ 6.1 (110) and <7.0 (126)
	ADA (2003)	FPG ≥ 5.6 (100) and <7.0 (126)
Impaired glucose tolerance (IGT)	WHO	FPG < 7.0 (126) + 2-h PG ≥ 7.8 and <11.1 (200)
Impaired glucose homeostasis (IGH)	WHO	IFG or IGT
Diabetes mellitus (DM)	WHO	FPG ≥ 7.0 (126) or 2-h PG ≥11.1 (200)
	ADA (1997)	FPG ≥ 7.0 (126)
	ADA (2003)	FPG ≥ 7.0 (126)

Values are expressed as venous plasma glucose.
FPG = fasting plasma glucose; 2-h PG = two-hour post-load plasma glucose (1 mmol/L = 18 mg/dL).

Table 2 Aetiological classification of glycaemia disorders[a]

- Type 1 (β-cell destruction, usually leading to absolute insulin deficiency)
  - Autoimmune
  - Idiopathic
- Type 2 (may range from predominantly insulin resistance with relative insulin deficiency to a predominantly secretory defect with or without insulin resistance)
- Other specific types (see *Table 3*)
  - Genetic defects of β-cell function
  - Genetic defects in insulin action
  - Diseases of the exocrine pancreas
  - Endocrinopathies
  - Drug- or chemical-induced
  - Infections
  - Uncommon forms of immune-mediated diabetes
  - Other genetic syndromes sometimes associated with diabetes, e.g.: Down's syndrome, Friedreich's ataxia, Klinefelter's syndrome, Wolfram's syndrome
- Gestational diabetes[b]

[a]As additional subtypes are discovered, it is anticipated that they will be reclassified within their own specific category.
[b]Includes the former categories of gestational IGT and gestational diabetes.

Table 3 Other specific types of diabetes

- Genetic defects of β-cell function
- Genetic defects in insulin action
  - e.g. Lipoatrophic diabetes
- Diseases of the exocrine pancreas
  - e.g. Pancreatitis, Trauma/pancreatectomy, Neoplasia, Cystic fibrosis
- Endocrinopathies
  - e.g. Cushing's syndrome, Acromegaly, Phaeochromocytoma, Hyperthyroidism
- Drug- or chemical-induced
  - e.g. cortisone, anti-depressant drugs, BBs, thiazide
- Infections
  - e.g. Cytomegalovirus
- Uncommon forms of immune-mediated diabetes

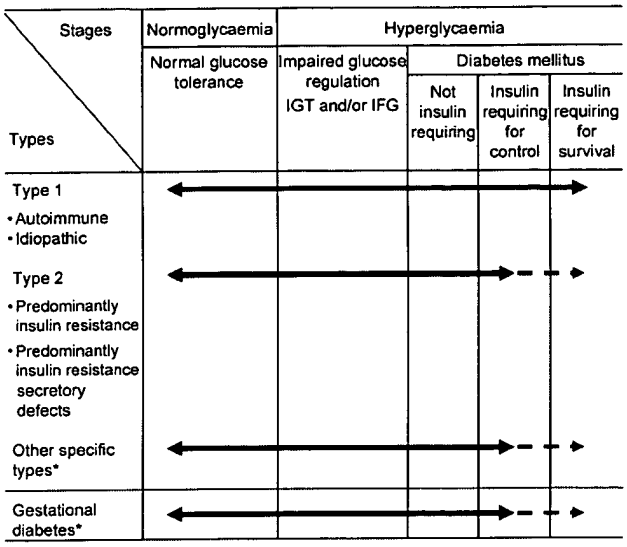


Figure 2 Disorders of glycaemia: aetiological types and clinical stages (see also *Table 3*).

individual may move from one category to another in either direction. Usually, a progression towards a more severe glucose abnormality takes place with increasing age. This is reflected by the increase in the 2-hPG level with age.[14]

The currently valid clinical classification criteria have been issued by WHO[4] and ADA.[7] These are currently under review by WHO and updated criteria will be introduced soon. The WHO recommendations for glucometabolic classification are based on measuring both fasting and 2-hPG concentrations and recommend that a standardized 75 g OGTT should be performed in the absence of overt hyperglycaemia.[4] The thresholds for diabetes on fasting and 2-hPG values were primarily determined by the values where the prevalence of diabetic retinopathy, which is a specific complication of hyperglycaemia, starts to increase. Even though macrovascular diseases such as CHD and stroke are major causes of death in type 2 diabetic patients and people with IGT, macrovascular disease has not been considered in the classification. This sounds illogical and may give an impression that macrovascular diseases are less important than microvascular consequences of diabetes. Classification according to the ADA criteria strongly encourages the single use of fasting glycaemia only without an OGTT.[6,7]

The currently recommended categories of glucose metabolism according to WHO and the ADA are presented in *Table 1* (for adults). The National Diabetes Data Group[2] and WHO[3] coined the term IGT, an intermediate category between normal glucose tolerance and diabetes. The ADA[6] and the WHO Consultation[4] proposed some changes to the diagnostic criteria for diabetes and introduced a new category called impaired fasting glucose/glycaemia (IFG). The ADA recently decreased the lower threshold for IFG from 6.1 to 5.6 mmol/L,[7] but this has been criticized and has not yet been adopted by the WHO expert group that recommends to keep the previous cut-points as shown in the WHO consultation report in 1999. These criteria were reviewed by a new WHO expert group in 2005.

In order to standardize glucose determinations, plasma has been recommended as the primary specimen. Since many equipment use either whole blood or venous or capillary blood, thresholds for these vehicles have also been given. The non-plasma recommendations for threshold are based on approximate estimates rather than on validated conversion factors. A recent analysis based on the direct pair-wise comparison of various types of specimens suggest that the factors presented in *Table 4* should be used to convert values measured in whole blood, capillary blood, and serum to plasma, respectively.[15]

The glucometabolic category in which an individual is placed depends on whether only fasting plasma glucose (FPG) is measured or if it is combined with a 2-hPG value. For example, an individual falling into the IFG category in the fasting state may have IGT or diabetes disclosed by a post-load glucose.

The metabolic determinants and physiological bases of FPG and 2-hPG differ to some extent.[1,16,17] This means the categorization of an individual on a FPG value may differ from that based on a 2-hPG. Having a normal FPG requires the ability to maintain an adequate basal insulin secretion and an appropriate hepatic insulin sensitivity to control

Table 4 Conversion factors between plasma and other vehicles for glucose values

Plasma glucose (mmol/L) = 0.558 + 1.119 × whole blood glucose (mmol/L)
Plasma glucose (mmol/L) = 0.102 + 1.066 × capillary blood glucose (mmol/L)
Plasma glucose (mmol/L) = −0.137 + 1.047 × serum glucose (mmol/L)

hepatic glucose output. Abnormalities of these functions characterize IFG. During an OGTT, the normal response to the absorption of the glucose load is both to suppress hepatic glucose output and to enhance hepatic and skeletal muscle glucose uptake. To keep a post-load glucose level within the normal range requires appropriate dynamics of the β-cell secretory response, amount and timing, in combination with adequate hepatic and muscular insulin sensitivity.

Recommendation
The definition and diagnostic classification of diabetes and its pre-states should be based on the level of the subsequent risk of cardiovascular complications. Class I, Level of Evidence B.

Glycated haemoglobin

Glycated haemoglobin (HbA_{1c}), a useful measure of metabolic control and the efficacy of glucose-lowering treatment, is an integrated summary of circadian blood glucose during the preceding 6–8 weeks, equivalent to the lifespan of erythrocytes.[18] It provides a mean value but does not reveal any information on the extent and frequency of blood glucose excursions. HbA_{1c} has never been recommended as a diagnostic test for diabetes.[4,7] A primary reason is the lack of a standardized analytical method and therefore lack of a uniform, non-diabetic reference level between various laboratories. A high HbA_{1c} may only identify a fraction of asymptomatic people with diabetes. HbA_{1c} is insensitive in the low range and a normal HbA_{1c} cannot exclude the presence of diabetes or IGT.

Markers of glucometabolic perturbations

An inherent difficulty in the diagnosis of diabetes is the present lack of an identified, unique biological marker that would separate people with IFG, IGT, or diabetes from people with normal glucose metabolism. The use of diabetic retinopathy has been discussed, but the obvious limitation is that this condition in a majority of the patients only becomes evident after several years of hyperglycaemic exposure.[1,5–10] On the other hand, diabetic retinopathy is diagnosed in ~1% of the non-diabetic population. Thus far, total mortality and CVD have not been considered for defining those glucose categories that carry a significant risk. Nevertheless, the vast majority of people with diabetes die from CVD and asymptomatic glucometabolic perturbations more than double mortality and the risk for myocardial infarction (MI) and stroke. Since the majority of type 2 diabetic patients develop CVD, which is a more severe (often even fatal) and costly complication of diabetes than retinopathy, CVD should be considered when defining cut-points for glucose.

Comparisons between FPG and 2-hPG

The diagnostic levels of FPG and 2-hPG are largely based on their association with the risk of having or to develop retinopathy. As outlined in the 1997 report by the ADA,[6] the incidence of retinopathy increases already above a FPG of ≥7.0 mmol/L, and not above the higher threshold level of 7.8 mmol/L as previously used for the diagnosis of diabetes. The DECODE Study (*Figure 3*) has shown that any mortality risk in people with elevated FPG is actually related to a concomitantly elevated 2-hPG glucose.[15,19,20] Thus, the current cut-off point for diabetes based on a 2-hPG ≥11.1 mmol/L may be too high. Lowering the threshold, although not yet formally challenged.

It has been noted that, although an FPG ≥7.0 mmol/L and a 2-hPG of ≥11.1 mmol/L sometimes identifies the same individuals, often they may not coincide. In the DECODE Study,[21] recruiting patients with diabetes by either criterion alone or by their combination, only 28% met both, and 40% met the fasting and 31% the 2-hPG criterion only (*Figure 4*). Among those who met the 2-hPG criterion, 52% did not meet the fasting criterion, and 59% of those who met the fasting criterion did not meet the 2-hPG criterion. In the U.S. NHANES III Study of previously undiagnosed diabetic adults aged 40–74 years, 44% met both the FPG and the 2-hPG criteria, whereas 14% met the FPG criterion only and 41% the 2-hPG criterion only.[22]

Screening for undiagnosed diabetes

Recent estimates suggest that 195 million people throughout the world have diabetes and that this number will increase to 330, maybe even to 500 million, by 2030.[23,24] Many patients, up to 50% in most investigations, with type 2 diabetes are undiagnosed[21,22,34] since they remain asymptomatic and therefore are undetected for many years. Detecting people with undiagnosed type 2 diabetes is important for both public health and every day clinical practice. Mass screening for asymptomatic diabetes has not been recommended in the general population pending evidence that the prognosis of such patients will improve by early detection and treatment.[25,26] Importantly, lack of evidence relates to lack of studies testing the hypothesis that early screening would indeed be advantageous. One such study (ADDITION) is ongoing in Denmark, the Netherlands, and the UK. Indirect evidence suggests that screening might be beneficial as it improves the possibility of early detection of diabetes and thereby improved prevention of cardiovascular complications. In addition, there is an increasing interest in identifying people with IGT, who might benefit from life style or pharmacological intervention to reduce or delay the progression to diabetes.[27]

Extensive data from epidemiological studies have challenged the practice not to utilize the 2-hPG showing that a substantial number of people, who do not meet the FPG criteria for abnormal glucose tolerance, will satisfy the criteria when exposed to an OGTT.[14,21,22,28] Thus, the risk of a false negative diagnosis is substantial when measuring FPG alone. The argument for FPG over 2-hPG is primarily related to the matter of feasibility. An OGTT has been considered a less

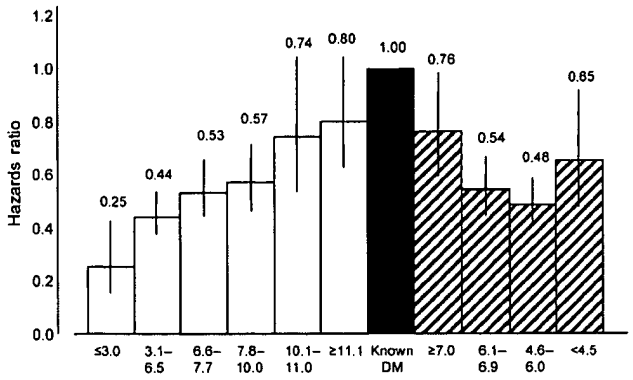


Figure 3 HR (columns) and 95%CI (vertical bars) for CVD mortality for fasting (FPG = striped bars) and 2-hPG (2hPG = open bars) intervals using previously diagnosed diabetes (black bar) as a common reference category. Data are adjusted for age, gender, cohort, BMI, systolic blood pressure, total cholesterol, and smoking (adapted from The DECODE Study Group[20]).

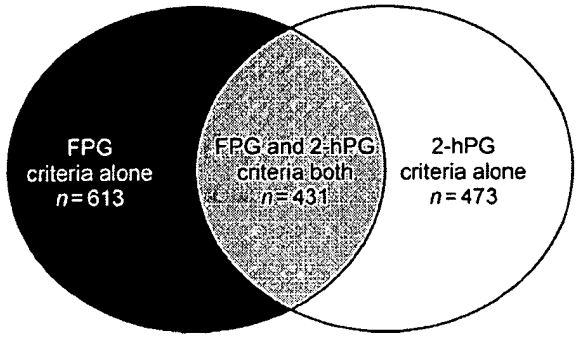


Figure 4 Fasting and post-load glucose identifies different individuals with asymptomatic diabetes. FPG = fasting plasma glucose; 2hPG = 2-h post-load plasma glucose (adapted from The DECODE Study Group[21]).

well-suited tool at a population level, mainly because the test takes somewhat more than 2 h to conduct. However, 2-hPG is the only way to detect IGT. Many subjects with IGT will develop CVD before progressing to diabetes.[28]

Recommendation
Early stages of hyperglycaemia and asymptomatic type 2 diabetes are best diagnosed by an OGTT that gives both fasting and 2-hPG values. Class I, Level of Evidence B.

Detection of people at high-risk for diabetes

Persons at high-risk for developing diabetes and those with asymptomatic diabetes by definition have no symptoms of diabetes and typically are not aware of their high-risk status. Although much attention has been directed at detecting undiagnosed type 2 diabetes in the past decades, only recently attention has turned to those with lesser degrees of glucometabolic abnormalities, which tend to share the same risk factors with type 2 diabetes.

Three general approaches for early detection exist: (i) measuring blood glucose to explicitly determine prevalent impaired glucose homeostasis (IGH), a strategy that will detect undiagnosed diabetes as well; (ii) using demographic and clinical characteristics and previous laboratory tests to determine the likelihood of future incident diabetes, a strategy that leaves current glycaemic state ambiguous; (iii) collecting questionnaire-based information on factors that provide information about the presence and extent of a number of aetiological factors for type 2 diabetes, a strategy that also leaves the current glycaemic state ambiguous.

The two latter approaches can serve as primary and cost-efficient screening tools, identifying a subgroup of the population in whom glycaemic testing may be targeted with a particular yield. The second option is particularly suited for certain groups, including those with pre-existing CVD and women who have had gestational diabetes, whereas the third option is better suited for the general population (*Figure 5*). Glycaemic testing is necessary as a secondary step in all three approaches to accurately define IGH, as the initial screening step is not diagnostic.

There will be a trade-off between sensitivity and specificity among the strategies. The final choice will depend on the goal and on relative health liabilities such as false positive vs. false negative. If the burden, as imposed by confirmatory testing, is not great and treatment is relatively harmless and inexpensive, one may accept a higher false positive rate. On the other hand, if the consequences of not treating in a timely manner are minor, a higher false negative rate may be acceptable. In algorithms that use multiple tests, the sequence will depend on the various steps leading to the confirmatory test, including costs, feasibility, and compliance. False labelling may be a problem in the first approach only, as the two other deals with elevated risk factors that are less sensitive to misclassification and by their own right already should lead to life style advise.[25] Including more glycaemic tests will contribute with more explicit information on the glycaemic status, whereas fewer tests result in more uncertainty. If a strategy does not incorporate an OGTT at any stage, individual glucose tolerance cannot be determined. Fasting glucose and HbA_{1c} will not reveal information about glucose excursions after meals or a glucose load.

It is necessary to separate three different scenarios: (i) general population; (ii) subjects with assumed metabolic abnormalities, including those who are obese, hypertensive, or who have a family history of diabetes; and (iii) patients with prevalent CVD. When patients with prevalent CVD have glucometabolic abnormalities, in most cases it is the 2-hPG value which is elevated, whereas fasting glucose is often normal.[30] Thus, the measurement of fasting glucose alone should be avoided in such patients. Since patients with CVD by definition can be considered at high-risk, there is no need to carry out a separate diabetes risk assessment, but an OGTT should be carried out in them. In the general population, the appropriate strategy is to start with risk assessment as the primary screening tool combined with subsequent glucose testing of individuals identified to be at a high risk.[31] This tool predicts the 10-year risk of type 2 diabetes with 85% accuracy, and in addition it detects current asymptomatic diabetes and abnormal glucose tolerance.[32,33]

Recommendation
Primary screening for the potential type 2 diabetes can be done most efficiently using a non-invasive risk score, subsequently combined with a diagnostic oral glucose tolerance testing in people with high score values. Class I, Level of Evidence A.

Finnish Diabetes Association

TYPE 2 DIABETES RISK ASSESSMENT FORM

Circle the right alternative and add up your points.

1. Age

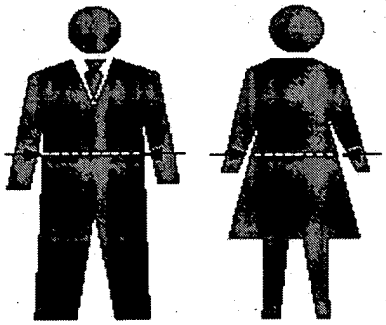
0 p. Under 45 years
2 p. 45–54 years
3 p. 55–64 years
4 p. Over 64 years

2. Body-mass index
(See reverse of form)

0 p. Lower than 25kg/m²
1 p. 25–30 kg/m²
3 p. Higher than 30 kg/m²

3. Waist circumference measured below the ribs (usually at the level of the navel)

	MEN	WOMEN
0 p.	Less than 94cm	Less than 80cm
3 p.	94–102cm	80–88cm
4 p.	More than 102cm	More than 88cm



4. Do you usually have daily at least 30 minutes of physical activity at work and/or during leisure time (including normal daily activity)?

0 p. Yes
2 p. No

5. How often do you eat vegetables, fruit or berries?

0 p. Every day
1 p. Not every day

6. Have you ever taken anti-hypertensive medication regularly?

0 p. No
2 p. Yes

7. Have you ever been found to have high blood glucose (e.g. in a health examination, during an illness, during pregnancy)?

0 p. No
5 p. Yes

8. Have any of the members of your immediate family or other relatives been diagnosed with diabetes (type 1 or type 2)?

0 p. No
3 p. Yes: grandparent, aunt, uncle or first cousin (but no own parent, brother, sister or child)
5 p. Yes: parent, brother, sister or own child

Total risk score

☐ The risk of developing type 2 diabetes within 10 years is

Lower than 7	Low: estimated 1 in 100 will develop disease
7–11	Slightly elevated: estimated 1 in 25 will develop disease
12–14	Moderate: estimated 1 in 6 will develop disease
15–20	High: estimated 1 in 3 will develop disease
Higher than 20	Very high: estimated 1 in 2 will develop disease

Please turn over

Test designed by Professor Jaakko Tuomilehto, Department of Public Health, University of Helsinki, and Jaana Lindström, MFS, National Public Health Institute.

Figure 5 FINnish Diabetes Risk SCore (FINDRISC) to assess the 10-year risk of type 2 diabetes in adults. Modified from ref. 31; available at: www.diabetes.fi/english.

Epidemiology of diabetes, IGH, and cardiovascular risk

Table of Recommendations:

Recommendation	Class[a]	Level[b]
The relationship between hyperglycaemia and CVD should be seen as a continuum. For each 1% increase of HbA_{1c} there is a defined increased risk for CVD	I	A
The risk of CVD for people with overt diabetes is increased by two to three times for men and three to five times for women when compared with people without diabetes	I	A
Information on post-prandial (post-load) glucose provides better information about the future risk for CVD than fasting glucose, and elevated post-prandial (post-load) glucose also predicts increased cardiovascular risk in subjects with normal fasting glucose levels	I	A
Improved control of post-prandial glycaemia may lower cardiovascular risk and mortality	IIb	C
Glucometabolic perturbations carry a particularly high risk for cardiovascular morbidity and mortality in women, who in this respect need special attention	IIa	B
People with diabetes and IGT have an increased risk for stroke	I	A
In stroke patients, unrecognized hyperglycaemia is mostly high post-load glucose seen in the OGTT, whereas the measurement of fasting glucose is insensitive in detecting unrecognized hyperglycaemia	I	B

[a]Class of recommendation.
[b]Level of evidence.

Introduction

The prevalence of type 2 diabetes increases with age especially in Europe.[14] Post-load hyperglycaemia reflects the acute increase in blood glucose after a glucose load, whereas fasting blood glucose shows the glucose concentration after an overnight fast and reflects mostly hepatic glucose production. They represent physiologically different aspects of glucose metabolism and may be differently influenced by the ageing process; post-prandial glucose excursions increase with age. The impact of gender on different abnormalities in glucose regulation is another unsolved issue.[23,35,36] Recently, the DECODE Study reported data on the age- and gender-specific prevalence of diabetes and IGH, as well as the age- and gender-specific prevalence of isolated fasting or 2-h post-load hyperglycaemia in European populations.[38]

Prevalence of diabetes and IGH

Plasma glucose concentrations, age and gender

The mean 2-h plasma glucose concentration rises with age in European populations, particularly after the age of 50 (*Figure 6*). Women have significantly higher mean 2-h plasma glucose concentrations than men, and this gender difference becomes more pronounced after the age of 70,

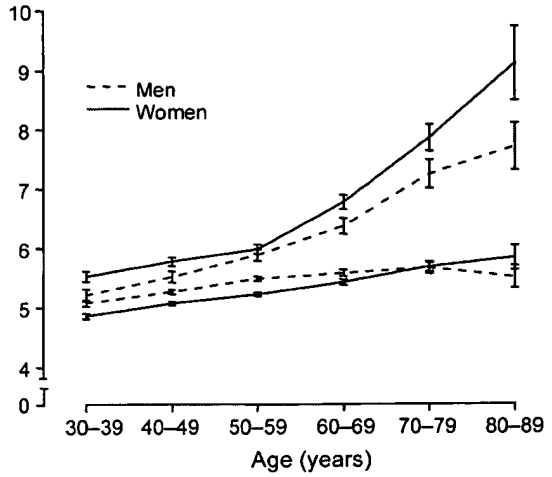


Figure 6 Mean fasting (the two lower lines) and 2-hPG (the two upper lines) concentrations and their 95%CI (vertical bars) in 13 European population-based cohorts included in the DECODE Study.[14]

probably because of survival disadvantage in men compared with women. Mean FPG concentration increases only slightly with age, in men up to 69 years and in women across all ages. Mean FPG concentration is higher in men than in women during the age period 30–69 years and becomes higher in women after 70 years.

Prevalence of diabetes and IGH

The age-specific prevalence of diabetes rises with age up to the seventh and eighth decades in both men and women (*Figure 7*).[14] The prevalence is less than 10% in subjects below the age of 60, 10–20% between 60–69 years, whereas 15–20% in the oldest age groups have previously known diabetes and a similar proportion have screen-detected asymptomatic diabetes. This suggests that the lifetime risk of diabetes in European people is 30–40%.

The prevalence of IGT increases linearly by age, but the prevalence of impaired fasting glycaemia does not (*Figure 8*). In middle-aged people, the prevalence of IGH is about 15%, whereas in the elderly 35–40% of European people have IGH. The prevalence of diabetes and IGT defined by isolated post-load hyperglycaemia is higher in women than in men, but the prevalence of diabetes and impaired fasting glucose (IFG) diagnosed by isolated fasting hyperglycaemia is higher in men than in women.[14]

Diabetes and coronary artery disease

The most common cause of death in European adults with diabetes is coronary artery disease (CAD). Several studies have demonstrated they have a risk that is two to three times higher than that among people without diabetes.[39] There are wide differences in the prevalence of CAD in patients with type 1[40] or 2 diabetes and also between different populations. The follow-up study of 10 centres of the WHO Multinational Study of Vascular disease in diabetes[41,42] including about 4700 type 1 and 2 patients, revealed that Japanese patients had a notably lower incidence of CAD than subjects from other parts of the world. Furthermore, their CAD incidence rates were lower than those in many

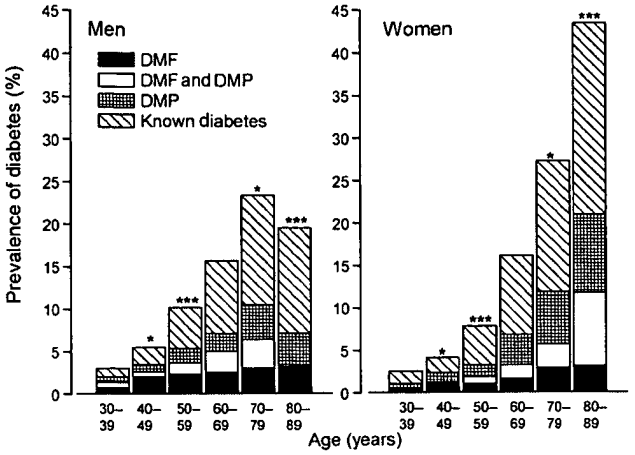


Figure 7 Age- and gender-specific prevalence of diabetes in 13 European population-based cohorts included in the DECODE Study.[14] DMF, diabetes determined by FPG ≥7.0 mmol/L and 2-h plasma glucose <11.1 mmol/L; DMP, diabetes determined by 2-h plasma glucose ≥11.1 mmol/L and FPG <7.0 mmol/L; DMF and DMP, diabetes determined by FPG ≥7.0 mmol/L and 2-h plasma glucose ≥11.1 mmol/L; known diabetes, previously diagnosed diabetes. $^{*}P < 0.05$; $^{***}P < 0.001$, for the difference in prevalence between men and women.

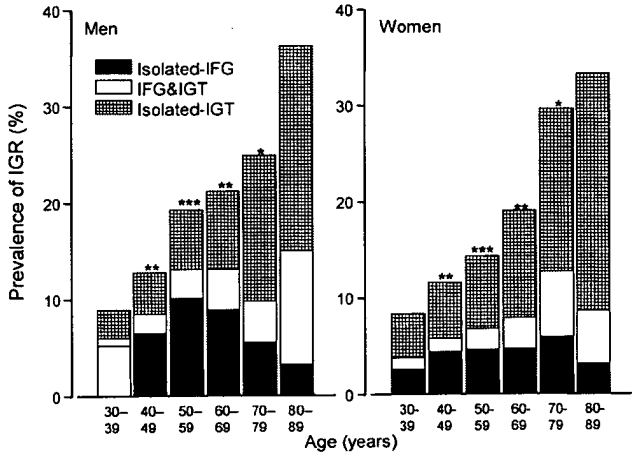


Figure 8 Age- and gender-specific prevalence of IGR in 13 European population-based cohorts included in the DECODE Study.[14] Isolated-IFG, FPG of 6.1-6.9 mmol/L and 2-h plasma glucose <7.8 mmol/L; isolated-IGT, 2-h plasma glucose of 7.8-11.0 mmol/L and FPG <6.1 mmol/L; IFG and IGT, FPG of 6.1-6.9 mmol/L and 2-h plasma glucose of 7.8-11.0 mmol/L. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, for the difference in prevalence between men and women.

non-diabetic western populations. CVD was the most common cause of mortality accounting for 44% of all deaths among patients with type 1 and 52% in patients with type 2 diabetes.[42] In the EURODIAB IDDM Complication Study, involving 3250 type 1 diabetic patients from 16 European countries, the prevalence of CVD (a past history and electrocardiographic abnormality) was 9% in men and 10% in women.[43] The prevalence increased by age, from 6% in the age group 15-29 years to 25% in the age group 45-59 years, and with the duration of diabetes. In type 1 diabetic patients, the risk of CAD increases dramatically with the onset of diabetic nephropathy. Up to 29% of patients with childhood-onset type 1 diabetes and nephropathy will, after 20 years with diabetes, have CAD compared to only 2-3% in similar patients without nephropathy.[44] In this context, besides hyperglycaemia, other CVD risk factors, such as hypertension, smoking, and dyslipidaemia, seem to be important contributing factors for CAD.[45,46]

Several studies compared the magnitude of risk for CAD associated with the history of type 2 diabetes or the presence of previous CAD. In a 7-year follow-up of a Finnish Study[47] and a 20-year follow-up of the Nurse's Health Study[48] patients with type 2 diabetes without any previous acute coronary events had a similarly high number of fatal CAD events as non-diabetic patients with a previous MI. The combination of type 2 diabetes and previous CAD identifies a group of patients with particularly high risk for coronary deaths. Moreover, the Nurse's Health Study indicated a strong relation between the duration of known diabetes and CAD mortality. Recently, data were reported from 51 735 Finnish men and women, aged 25-74 years and followed for an average of 17 years, during which time 9201 deaths occurred.[49] Among men with diabetes only, with MI only and with both diseases, combined hazard ratios (HR) for coronary mortality, adjusted for other risk factors, were 2.1, 4.0, and 6.4, respectively, compared to men without either disease. The corresponding HRs for women were 4.9, 2.5, and 9.4. HRs for total mortality were 1.8, 2.3, and 3.7 in men and 3.2, 1.7, and 4.4 in women. Diabetic men and women had comparable mortality rates, whereas coronary mortality among men was markedly higher. Thus, a history of diabetes and MI markedly increased CVD and all-cause mortality. The relative effect of diabetes was larger in women, whereas the relative effect of the history of MI was more substantial among men. The increased risk of CAD in subjects with diabetes was only partly explained by concomitant risk factors including hypertension, obesity, dyslipidaemia, and smoking. Thus, the diabetic state or hyperglycaemia itself and its consequences are very important for the increased risk for CAD and related mortality. Further support to the important relation between diabetes and MI was obtained from the Interheart Case Control Study.[160] Diabetes increased the risk by more than two times in men and women independent of ethnicity.

Asymptomatic hyperglycaemia and CAD

In 1979, a series of papers from the International Collaborative Group[50] did not find any consistent evidence for either a threshold or a graded association between asymptomatic hyperglycaemia and CAD. There were, however, several methodological concerns with these early studies. Many of them used fasting glucose only; moreover, differences in glucose assays, glucose load, sample time after loading, follow-up time, and the population studied may have contributed to the inconsistent observations. After the introduction of standard criteria, in 1980, several studies revealed an association between 2-h plasma glucose and CAD in the general population.[51-63] Some studies also showed an association with fasting glucose. A meta-analysis of 20 epidemiological studies found a progressive relationship between plasma glucose, fasting and post-load, and the incidence of cardiovascular events among people without diabetes. However, the results were not adjusted for other potential confounding factors.[64]

Recommendation
The relationship between hyperglycaemia and CVD is to be seen as a continuum. For each 1% increase of HbA_{1c}, there is a defined increased risk for CVD. Class I, Level of Evidence A.

The risk of CVD for people with overt diabetes is increased by two to three times for men and three to five times for women compared to people without diabetes. Class I, Level A.

IGH and CAD

Cardiovascular risk and post-prandial hyperglycaemia

The major disagreement in the classification of glucose homeostasis between the criteria issued by WHO and ADA focuses on whether diabetes should be diagnosed by means of a fasting or a 2-hPG. While different people are identified as diabetic and particularly as having IGH, when testing for fasting glucose than for a post-load glucose, it is clinically important to know how these two entities relate to mortality and the risk for CVD. Three early cohort studies, the Whitehall Study, the Paris Prospective Study, and the Helsinki Policemen Study, assessed the relationship between 2-hPG and the risk for CAD in European men.[56,57,65] With known diabetes excluded, CVD mortality in individuals with a high 2-hPG (>95th centile in the Whitehall Study and >80th centile in the Paris and Helsinki studies) was twice that in subjects with normal glucose levels. In the Japanese Funagata Diabetes Study, survival analysis concluded that IGT, but not IFG was a risk factor for CVD.[63] In a recent Finnish Study, IGT at baseline was an independent risk predictor of incident CVD and premature all-cause and cardiovascular mortality, a finding not confounded by the development of clinically diagnosed diabetes during follow-up.[29]

The 23-year follow-up of the Honolulu Heart Programme suggested a dose-response relationship between 1 h glucose after a 50 g load and CAD mortality.[59] The Chicago Heart Study of ~12 000 men without a history of diabetes showed that white men with asymptomatic hyperglycaemia [1 h glucose ≥11.1 mmol/L (200 mg/dL)] had an increased risk of CVD mortality compared with men having a low post-load glucose <8.9 mmol/L (160 mg/dL).[58] The Rancho Bernardo Study indicated that elderly Californian women (but not men) with isolated post-challenge hyperglycaemia [2-hPG ≥11.1 mmol/L (200 mg/dL) and FPG <7.0 mmol/L (126 mg/dL)] had a significantly increased risk of CVD.[51]

Several studies assessed the association of CVD with fasting and 2-hPG. Based on longitudinal studies in Mauritius, Fiji, and Nauru, Shaw *et al.*[62] reported that people with isolated post-challenge hyperglycaemia doubled their CVD mortality compared with non-diabetic persons, whereas there was no significant increase in mortality related to isolated fasting hyperglycaemia [FPG ≥7.0 mmol/L (126 mg/dL) and 2-hPG <11.1 mmol/L (200 mg/dL)]. In the Cardiovascular Health Study, including 4515 subjects above the age of 65 years, the relative risk for incident CAD was higher in individuals with abnormal glucose homeostasis (comprising IGT, IFG, and newly diagnosed diabetes, detected by both fasting and 2-hPG) than in those with normal glucose levels. However, criteria based on FPG alone were less sensitive than the WHO 1999 criteria based on fasting and 2-hPG for predicting CAD.[52] A recent analysis of the US Second National Health and Nutrition Survey data, including 3092 adults aged 30–74 years, found a graded increase in mortality associated with abnormal glucose tolerance ranging from a 40% greater risk in adults with IGT to an 80% greater risk in adults with newly diagnosed diabetes.[67]

The most convincing evidence for a relation between abnormal glucose tolerance and an increased CAD risk has been provided by the DECODE Study, jointly analysing data from more than 10 prospective European cohort studies including more than 22 000 subjects.[68,69] Death rates from all-causes, CVD, and CAD were higher in diabetic subjects diagnosed by 2-hPG than in those not meeting this criterion. Significantly increased mortality was also observed in subjects with IGT, whereas there was no difference in mortality between subjects with impaired and normal fasting glucose. Multivariate analyses showed that high 2-hPG predicted mortality from all-causes, CVD, and CAD, after adjustment for other major cardiovascular risk factors, but high fasting glucose alone did not. High 2-hPG was a predictor for death, independent of FPG, whereas increased mortality in people with elevated FPG largely related to the simultaneous elevation of the 2-hPG. On the other hand, FPG did not add any predictive information once 2-hPG was entered into the model. All-cause and CVD mortality were increased in subjects with an FPG ≥7.0 mmol/L (126 mg/dL), but even among them it was a simultaneous elevation of 2-hPG that explained the increased mortality.[15,19] The largest absolute number of excess CVD mortality was observed in subjects with IGT, especially those with normal FPG. The relation of 2-hPG with mortality was linear, but such a relation was not seen with FPG.

Recommendation
Information on post-prandial (post-load) glucose provides better information about the future risk for CVD than fasting glucose, and elevated post-prandial glucose also predicts the cardiovascular risk in subjects with normal fasting glucose levels. Class I, Level of Evidence A.

Glycaemic control and cardiovascular risk

Although several prospective studies have unequivocally confirmed that post-load hyperglycaemia increases CVD morbidity and mortality and is a better predictor for subsequent events than a high FPG, it still remains to be demonstrated that lowering a high 2-hPG will reduce this risk in well designed, randomized controlled trials (RCT). Such studies are underway, but thus far data are scarce. A secondary endpoint analysis of the STOP-NIDDM (Study TO Prevent Non-Insulin-Dependent Diabetes Mellitus) revealed statistically significant reductions in CVD event rates in IGT subjects receiving acarbose compared with placebo.[70] Since acarbose specifically reduces post-prandial glucose excursions, this is the first demonstration that lowering post-prandial glucose may lead to a reduction in CVD events. It should, however, be noted that the power in this analysis is low due a very small number of events.

The largest trial in type 2 diabetic patients so far, the United Kingdom Prospective Diabetes Study,[71] was not powered to test the hypothesis that lowering blood glucose by intensive treatment can reduce the risk of MI, although there was a 16% (marginally significant) reduction in intensively compared with conventionally treated

patients. In this study, post-load glucose excursions were not measured and over the 10 years of follow-up, the difference in the HbA_{1c} concentrations between the intensive and conventional groups was only 0.9% (7.0 vs. 7.9%). Moreover, the drugs used for intensive treatment, sulphonylureas, long-acting insulin and metformin, mainly influence FPG, but not post-prandial glucose excursions. The German Diabetes Intervention Study, recruiting newly diagnosed type 2 diabetic patients, is so far the only intervention study that has demonstrated that controlling post-prandial hyperglycaemia (blood glucose measured 1 h after breakfast) had a greater impact on CVD and all-cause mortality than controlling fasting blood glucose.[72] During the 11-year follow-up, poor control of fasting glycaemia did not significantly increase the risk of MI or mortality, whereas poor vs. good control of post-prandial glucose was associated with a significantly higher mortality. Additional support is obtained from a meta-analysis of seven long-term studies using acarbose in type 2 diabetic patients. The risk for MI was significantly lower in patients receiving acarbose compared with those on placebo.[73]

Recommendation
Improved control of post-prandial glycaemia may lower cardiovascular risk and mortality. Class IIb, Level of Evidence C.

Gender difference in CAD related to diabetes

In the middle-aged general population, men have a two to five times higher risk for CAD than women.[74,75] The Framingham Study was the first to underline that women with diabetes seem to lose their relative protection against CAD compared with men.[76] The reason for the higher relative risk of CAD in diabetic women than diabetic men is still unclear.

The 14-year follow-up of the Rancho Bernardo Study showed that the multivariate-adjusted relative hazards of death from CAD in diabetic, compared with non-diabetic subjects, was 3.3 in women and 1.9 in men.[77] In a 13-year follow-up study of a Finnish cohort, free from CVD at baseline and with or without type 2 diabetes, the diabetes-related adjusted HR for a major coronary event was 2.8 (95%CI 2.0–3.7) for men and 9.5 (95%CI 5.5–16.9) for women.[78] In a Scottish 12-year long follow-up, asymptomatic hyperglycaemia (casual blood glucose >7.0 mmol/L) was a significant risk factor for CVD in both genders, however, it was a stronger risk factor in women than in men.[79] A review about the impact of gender on the occurrence of CAD mortality reported that the overall relative risk (the ratio of men to women) for CAD mortality was 1.46 (95%CI 1.21–1.95) in diabetic and 2.29 (2.05–2.55) in non-diabetic subjects. This suggests that the gender differential is reduced in diabetes.[35] The result from the DECODE Study, including 8172 men and 9407 women without known diabetes, showed that newly diagnosed diabetic women had a higher relative risk for cardiovascular mortality than newly diagnosed diabetic men.[68] This association was independent of age, body mass index (BMI), systolic blood pressure, total cholesterol, and smoking. Recent data related to hormonal replacement therapy show that, particularly in diabetic women, the risk of CVD increases significantly.[80]

A meta-analysis of 37 prospective cohort studies, including 447 064 diabetic patients estimated the diabetes-associated, gender-related risk of fatal CAD.[81] CAD mortality was higher in patients with diabetes than in those without (5.4 vs. 1.6%). The overall relative risk among people with and without diabetes was significantly greater among women with diabetes 3.50 (95%CI 2.70–4.53) than among men with diabetes 2.06 (95%CI 1.81–2.34).

Recommendation
Glucometabolic perturbations carry a particularly high risk for cardiovascular morbidity and mortality in women, who in this respect need special medical attention. Class IIa, Level of Evidence B.

Glucose homeostasis and cerebrovascular disease

Diabetes and stroke

The risk for cerebrovascular morbidity and mortality (stroke, cerebrovascular accidents), which causes substantial costs for society, is magnified by diabetes.[82–89] Indeed, CVD is the predominant long-term cause of morbidity and mortality in patients with both type 1 and type 2 diabetes. Since the first observations, presented by the Framingham investigators, several large population-based studies have verified an increased frequency of stroke in the diabetic population.[85,88] Diabetes was the strongest single risk factor for stroke (relative risk for men 3.4 and for women 4.9) in a prospective study from Finland with a follow-up of 15 years.[82] Diabetes is a prominent risk factor for ischaemic stroke, but data on haemorrhagic stroke have been controversial,[90–93] although a recent report from the Framingham Study suggested an increased risk of haemorrhagic stroke in type 2 diabetes.[88]

In Europe, ischaemic CVD accounts for about 80% of all strokes,[94] but the female:male mortality ratio differs for stroke subtypes, ethnicity, and age.[93,94] DM may also cause microatheromas in small vessels, such as the lenticulostriate arteries, leading to lacunar stroke, one of the most common subtypes of ischaemic stroke. Lacunar stroke is a unique subtype and requires specific clinical and imaging features for diagnosis. The presence of DM was associated with symptomatic cerebral infarcts, but not with silent infarcts,[95] which are five times as prevalent as symptomatic brain infarcts in the general population.[96]

An inverse correlation has been reported between diabetes and aneurysmal subarachnoid haemorrhage, but two studies claim that diabetes is closely associated with subarachnoid haemorrhage.[97,98] Stroke patients with diabetes, or with hyperglycaemia in the acute stage of stroke, have a higher mortality, worse neurological outcome, and more severe disability than those without.[82,90–101]

There is much less information concerning the risk of stroke in type 1 than in type 2 diabetes. Deckert *et al.*[102] who followed type 1 diabetic patients for more than 40 years, reported a 10% cumulative incidence of stroke and 7% mortality from stroke. The World Health Organization Multinational Study of Vascular Disease in Diabetes indicated increased cerebrovascular mortality in type 1 diabetic patients, however, with considerable variations between countries.[103] Patients, generally, are much younger in type 1 than in type 2 diabetes, and stroke is known as a disease of elderly people with two-thirds of all strokes occurring above the age of 65 years. Thus, the true risk of stroke in type 1 diabetic patients may still be less well-established.

The data from the nationwide cohort of more than 5000 Finnish childhood-onset type 1 diabetic patients showed that, by the age of 50 years (i.e. after 20–40 years with diabetes), the risk for an acute stroke was equal to that of an acute coronary event without any gender-related difference.[44] Presence of diabetic nephropathy was the strongest predictor of stroke, causing a 10-fold increase of risk.

IGT and stroke

Considerably less is known about the frequency of asymptomatic diabetes and IGT in patients with stroke. In a recent Austrian study[104] involving 238 patients, 20% had previously known diabetes, 16% newly diagnosed diabetes, 23% IGT, but only 0.8% had IFG. Thus, as few as 20% had a normal glucose homeostasis. Another 20% of the patients had hyperglycaemic values, which could not be fully classified due to missing data in the OGTT. Patients with diabetes had more severe strokes at admission, a more serious outcome at discharge, and a higher rate of infectious complications. In an Italian study, 106 patients were recruited with acute ischaemic stroke and without any history of diabetes, 81 patients (84%) had abnormal glucose metabolism at discharge and 62 (66%) after 3 months (39% IGT and 27% newly detected diabetes). Post-load hyperglycaemia at discharge was a predictor of diabetes after 3 months.[105]

Recommendation

People with diabetes and IGT have an increased risk for stroke. Class I, Level of Evidence A.

In stroke patients, unrecognized hyperglycaemia is mostly high post-load glucose seen in the OGTT, whereas the measurement of fasting glucose is insensitive in detecting unrecognized hyperglycaemia. Class I, Level of Evidence B.

Prevention of CVD in people with IGH

Although overall trends in CVD mortality have shown a significant downward trend in developed countries during the last decades, it has been suggested that the decline has been smaller or not existent at all in diabetic subjects.[106] A more recent study reports on a 50% reduction in the rate of incident CVD events among adults with diabetes. The absolute risk of CVD was, however, two-fold greater than among persons without.[161] More data are needed to judge this issue in European populations.

Accumulating evidence has shown that deterioration of IGT in type 2 diabetes can be effectively prevented by life style intervention.[22,109] Whether this also leads to the prevention of CVD needs to be seen in the follow-up of these trial populations.

An imminent issue is to prove that prevention and control of post-prandial hyperglycaemia will cause a reduction in mortality, CVD, and other late complications of type 2 diabetes. There is also a need to reconsider the thresholds used to diagnose hyperglycaemia.[20] The majority of premature deaths related to IGH occur in people with IGT[15,19] obviating the need for increased attention to people with a high 2-hPG. A first step would be to detect such people through systematic screening of high-risk groups (see the following section).[31,41] The best way to prevent the negative health consequences of hyperglycaemia may be to prevent the development of type 2 diabetes. Controlled clinical outcome trials among asymptomatic subjects with hyperglycaemia are underway, but results will only be available after some years. Meanwhile, the only way to make clinical treatment decisions in such subjects is to make inferences from the observational epidemiological data and pathophysiological studies.

Identification of subjects at high risk for CVD or diabetes

Table of Recommendations:

Recommendation	Class[a]	Level[b]
The metabolic syndrome identifies people at higher risk of CVD than the general population, although it may not provide a better or even equally good prediction of cardiovascular risk than scores based on the major cardiovascular risk factors (blood pressure, smoking, and serum cholesterol)	II	B
Several cardiovascular risk assessment tools exist and they can be applied to both non-diabetic and diabetic subjects	I	A
An assessment of predicted type 2 diabetes risk should be part of the routine health care using the risk assessment tools available	II	A
Patients without known diabetes but with established CVD should be investigated with an OGTT	I	B
People at high risk for type 2 diabetes should receive appropriate life style counselling, and if needed, pharmacological therapy to reduce or delay their risk of developing diabetes. This may also decrease their risk to develop CVD	I	A
In people with IGT, the onset of diabetes can be delayed by certain drugs (such as metformin, acarbose and rosiglitazone)	I	A
Diabetic patients should be advised to be physically active in order to decrease their cardiovascular risk	I	A

[a]Class of recommendation.
[b]Level of evidence.

The metabolic syndrome

Longitudinal clinical and epidemiological studies consistently demonstrated that certain risk factors are important as causes of the mass occurrence of CVD in populations and also serve as contributing factors to increased risk at an individual level. These risk factors include dyslipidaemia, hyperglycaemia and diabetes, hypertension and factors linked to unhealthy life styles such as unhealthy eating habits, physical inactivity, overweight/abdominal obesity, and smoking. A family history of premature cardiovascular events, probably reflecting both genetic and environmental factors, has also been considered important in defining cardiovascular risk.

The clustering of metabolic and physiological abnormalities was noticed as early as 1923 by Kylin, who described a syndrome consisting of the co-existence of hypertension, hyperglycaemia, and hyperuricaemia.[113] In 1947, Vague paid attention to the influence of body fat distribution on the development of metabolic abnormalities,[114] but the real interest in clustering metabolic and physiological abnormalities started in the 1980s.[115-117] In 1988, Reaven described a syndrome based on the clustering of the following abnormalities: resistance to insulin-stimulated glucose uptake, hyperinsulinaemia, hyperglycaemia, increased very low-density lipoprotein (VLDL) triglycerides, decreased high-density lipoprotein (HDL) cholesterol, and high blood pressure.[118] Since then, there has been a growing interest in the clustering of factors, each one associated with increased risk for CVD. Subsequently, this syndrome became referred to as the 'insulin-resistance syndrome'[119] or the 'metabolic syndrome'.[120] The role of obesity and its central distribution has become a subject of some debate. In his original description, Reaven considered that obesity did not belong to the syndrome, although it could contribute to its development.[118] More recently, several new components have been proposed as belonging to the syndrome, including markers of inflammation, microalbuminuria, hyperuricaemia, and fibrinolytic and coagulation abnormalities.[121]

Definitions

Currently, there are at least five definitions of the metabolic syndrome proposed by international or national organizations or expert groups: the World Health Organization (WHO) in 1998[122] revised in 1999;[4] the European Group for Study of Insulin Resistance (EGIR) in 1999;[124,125] the National Cholesterol Education Programme (NCEP) Adult Treatment Expert Panel III in 2001;[126,127] the American Association of Clinical Endocrinologists (AACE) in 2003;[128,129] and more recently International Diabetes Federation (IDF) Consensus Panel[130] http://www.idf.org/webdata/docs/Metabolic_syndrome_definition.pdf. The WHO and EGIR definitions were primarily proposed for research purposes and the NCEP and AACE definitions for clinical use. The 2005 IDF definition aims at worldwide clinical practice. Tables listing the various definitions are presented in the section on pathophysiology.

Studies on the relationship between the presence of metabolic syndrome and the risk of mortality and morbidity are still scarce, particularly, the comparison of risk by different definitions of the syndrome. Several studies in Europe revealed that the presence of the metabolic syndrome increased CVD and all-cause mortality,[131-134] but a couple of reports from the US have shown inconsistent evidence. The Strong Heart Study[135] followed up, for over 7.6 years, 2283 American Indians who were free of CVD and diabetes at baseline examination. The incidence of diabetes increased by the presence of the NCEP syndrome, but that of CVD did not. Age-and centre-adjusted HR for having the NCEP metabolic syndrome was 1.13 (95%CI 0.81-1.58) in non-diabetic participants. Based on the data of 2431 US adults aged 30-75 years participating in the second National Health and Nutrition Examination Survey (NHANES II), it was found that the metabolic syndrome was associated with a moderately increased risk of mortality from CVD, but not significantly associated with mortality from all-causes, CHD, or stroke.[136] In the San Antonio Heart Study, after excluding subjects with diabetes, the corresponding relative risk for all-cause mortality decreased substantially from 1.45 (1.07-1.96) to 1.06 (0.71-1.58) for the NCEP definition and from 1.23 (0.90-1.66) to 0.81 (0.53-1.24) for a modified WHO syndrome.[137] A recent study revealed that the NCEP metabolic syndrome is inferior to established predictive models for either type 2 diabetes or CVD.[138] Lawlor *et al.* recently showed that point estimates of the effect for each definition of the syndrome were similar as or even weaker than those for individual factors, suggesting there is little additional prognostic value in defining the individual factors as a syndrome for predicting CVD mortality.[139] Although each definition of the metabolic syndrome includes several risk factors they are defined dichotomously. Thus, such a prognostic formula cannot predict CVD as accurately as a risk model based on continuous variables.

Recommendation

The metabolic syndrome identifies people at a higher risk of CVD than that in the general population, although it may not provide a better or even equally good prediction of cardiovascular risk than scores based on the major cardiovascular risk factors (age, blood pressure, smoking, and serum cholesterol). Class II, Level of Evidence B.

Risk charts

Various risk charts or scores have been developed to assess the risk for non-fatal or fatal cardiovascular events within a given time frame in individuals without a previous cardiovascular diagnosis. The first of these, the Framingham risk score, has been available since 1967 in the US, comprising the major risk factors known by that time: gender, age, systolic blood pressure, total cholesterol, cigarette smoking, and diabetes. The most recent Framingham score added HDL-cholesterol and deleted left ventricular (LV) hypertrophy.[140] The Framingham and other risk scores have been tested in different populations[141-149] and the conclusion from the comparative studies is that, while the absolute risk may differ from population to population, the proportionate risk ranking provided by these scores is consistent across populations.

A European Heart Score has been generated based on pooled data from more than 200 000 men and women,[150] which takes the overall CVD risk profile into account. It does, however, only include the traditional risk factors: age, gender, total cholesterol, smoking, and systolic blood pressure. This risk score is based on fatal events. Diabetes has not yet been taken into account as it was not uniformly defined in the cohorts based upon which this score was developed. Future attempts will be made to include diabetes and 2-hPG.

Recommendation

Several cardiovascular risk assessment tools exist and they can be applied to both non-diabetic and diabetic subjects. Class I, Level of Evidence A.

Diabetes is a major risk factor for CVD

In the EUROASPIRE II cohort, 29% of all coronary patients had known diabetes and another 23% IGT.[151] The mortality follow-up of the EUROASPIRE I cohort showed that, apart from smoking, diabetes is the most important, single risk

factor for total and cardiovascular mortality in these coronary patients.[152] Results from a number of cohort studies, particularly the large European DECODE Study, indicate that either fasting or 2-hPG are independent risk factors for all-cause and cardiovascular morbidity and mortality even in people without diagnosed diabetes.[15,19,20,69] More recently, the DECODE group developed a CVD risk score, which presently is the only one of its kind including IGT or IFG in the risk function determination.[157]

Risk functions are usually estimated quantitatively depending on the characteristics of the studied population and the risk factors that have been included. Each population may have a different distribution of risk factors, which may weigh differently in determining the disease and the disease may occur with different probability. Moreover, there must be secular differences between different generational cohorts within one population. Therefore, the predictive accuracy of a risk score may be only adequate for the index population. It is a challenge to know whether a worldwide uniform CVD risk assessment tool can be developed.

A population strategy for altering life style and environmental factors, the underlying causes of the mass occurrence of CAD, has been considered since 1982 in a report of the WHO Expert Committee on Prevention of Coronary Heart Disease. This is in accordance with the notion that even small decreases in the risk factor pattern at a population level, through the large number of individuals involved, will affect the health of many people.[158] Such an approach has proven successful in Finland.[159] There is now a need to develop a CVD risk assessment tool based on easily available information and intended for public health purposes similar to the one developed to predict the development of type 2 diabetes in Finland.[32] This Finnish Diabetes Risk Score (FINDRISC) (see the section on Detection of people at high-risk for diabetes; *Figure 5*) predicts the 10-year risk for developing type 2 diabetes with 85% accuracy. It also detects asymptomatic diabetes and abnormal glucose tolerance with high reliability in other populations.[32,111] In addition to the prediction of diabetes, FINDRISC predicts the incidence of MI and stroke.[163] Such high-risk individuals identified by a simple scoring system can be a target for proper management, not only for diabetes prevention, but at the same time, for CVD prevention.

Recently, the definition of the NCEP metabolic syndrome and the Framingham cardiovascular risk score were compared for the prediction of cardiovascular events. Data from the population-based San Antonio Study[138] showed that the Framingham risk score predicted CVD better than the metabolic syndrome. This is not surprising considering that the Framingham score, in contrast to the metabolic syndrome, was specifically developed to predict cardiovascular events and that it differs by including smoking as a risk factor. In the Atherosclerosis Risk in Communities Study ARIC cohort,[164] the metabolic syndrome was found to be predictive of cardiovascular events, both in men and women, but as in the San Antonio Study, the inclusion of the metabolic syndrome did not improve the coronary risk prediction beyond what was achieved with the Framingham risk score. Nevertheless, neither the definition of the metabolic syndrome nor any of the CVD risk scores are perfect with regard to the accuracy of risk prediction.[165-169]

Recommendation

An assessment of predicted type 2 diabetes risk should be part of the routine health care using the risk assessment tools available. Class II, Level of Evidence A.

Patients without known diabetes but with established CVD should be investigated with an OGTT. Class I, Level of Evidence B.

Preventing progression to diabetes

The development of type 2 diabetes is often preceded by a variety of altered metabolic states, including IGT, dyslipidaemia, and insulin resistance.[170] Although not all patients with such metabolic abnormalities progress to diabetes, their risk of developing the disease is significantly enhanced. Life style factors, such as poor diet and a sedentary life style, which in turn lead to obesity, have a major impact on the risk of developing type 2 diabetes.[171-173] Carefully conducted clinical studies[174-178] have demonstrated that effective life style intervention strategies and drug treatments can prevent or at least delay the progression to type 2 diabetes in high-risk individuals.

The Swedish Malmö Study used increased physical exercise and weight loss as major intervention strategies to prevent or delay type 2 diabetes.[174] Subjects with IGT had less than half the risk of developing type 2 diabetes compared with those who did not take part in the exercise programme during 5 years of follow-up.

In a Chinese study from Da Qing, 577 individuals with IGT were randomized by clinic into one of the four groups: exercise only, diet only, diet plus exercise, and a control group.[175] The cumulative incidence of type 2 diabetes during 6 years was significantly lower in the three intervention groups compared with the control group (41% in the exercise group, 44% in the diet group, 46% in the diet plus exercise group, and 68% in the control group) and this difference remained significant after adjusting for differences in baseline BMI and fasting glucose.

The Finnish Diabetes Prevention Study found that a reduction in bodyweight achieved through an intensive diet and exercise programme was associated with a 58% reduction in the risk of developing type 2 diabetes ($P < 0.001$).[108] Middle-aged men ($n = 172$) and women ($n = 350$) who were overweight and had IGT were randomized to the intervention group or to a control group who had conventional care. The goals of the life style interventions were to achieve a $\geq$5% reduction in bodyweight, reduce all fat intake to less than 30% of energy consumption, particularly saturated fat less than 10% of energy consumption, increase fibre intake of at least 15 g/1000 kcal, and undertake a programme of moderate exercise for 30 min/day or more.[179] After 2 years, patients in the intervention group had achieved a significantly greater mean reduction in body-weight compared with those in the control group ($P < 0.001$). They also demonstrated favourable changes in measures of glycaemia, including improved fasting and post-challenge plasma glucose levels. In addition, fewer subjects in the intervention group developed type 2 diabetes than in the control group [58% relative risk reduction (RRR); $P < 0.001$]. The reduction in the risk of progression to diabetes was directly related to the magnitude of the changes in life style; none of the patients who had achieved at least four of the intervention goals by 1 year developed type 2 diabetes during follow-up.[108,179]

The U.S. Diabetes Prevention Programme also found that life style modification reduced the incidence of type 2 diabetes by 58% in overweight American adults with IGT.[109] A total of 3234 adults (1043 men and 2191 women) were randomized to standard life style recommendations plus placebo or metformin 850 mg twice daily, or to an intensive life style modification programme. The goal of the programme was to achieve and maintain ≥7% reduction in body weight, through a low-calorie, low-fat diet plus physical activity of moderate intensity for at least 150 min per week. Patients in the life style intervention group had a significantly greater mean reduction in body weight (5.6 kg, $P < 0.001$) compared with those in the placebo (0.1 kg) and metformin groups (2.1 kg). The cumulative incidence of diabetes during the follow-up period was lower in the life style intervention and metformin groups than in the placebo group, with incidence rates of 4.8, 7.8, and 11.0 cases per 100 person-years, respectively. This reduction in incidence equated to one case of diabetes prevented for every seven people with IGT treated for 3 years in the life style intervention group, compared with 14 of the metformin group.

The STOP-NIDDM Trial was a double-blind, placebo-controlled, randomized trial comprising 1429 subjects with IGT.[178] The α-glucosidase inhibitor acarbose was used as an active agent in this trial with the primary aim to determine whether the incidence of diabetes could be prevented. The risk of progression to diabetes over 3.3 years was reduced by 25%. Furthermore, acarbose increased the probability of IGT reverting to normal glucose tolerance over time.

In light of the impressive results of the Finnish Diabetes Prevention Study and the Diabetes Prevention Programme, the ADA and the National Institutes of Diabetes, Digestive, and Kidney Diseases (NIDDK) recommend that people over 45 years with BMI ≥25 kg/m^2 should be screened for evidence of high blood glucose. People found to have evidence of a pre-diabetic state should be given appropriate counselling on the importance of weight loss through a programme of dietary modification and exercise.[180] In addition, since patients with the metabolic syndrome have an increased risk of CVD and mortality,[131,132,136] life style interventions in obese patients and those with evidence of obesity or hyperglycaemia are likely to be beneficial in terms of overall health and life expectancy. The numbers needed to treat (NNT) to prevent one case of type 2 diabetes with life style intervention in people with IGT is dramatically low (*Table 5*). Thus, the benefits from the life style intervention are great. In the recently reported Indian Diabetes Prevention Programme (IDPP), life style and metformin showed similar capability to reduce the incidence of diabetes, but a combination of these two treatment possibilities did not improve the outcome.[37]

The Diabetes REduction Assessment with ramipril and rosiglitazone Medication (DREAM)[268,318] trial investigated prospectively whether these two pharmacological compounds may reduce the onset of diabetes, using a factorial design, in people with impaired glucose tolerance, impaired fasting glucose or both. The primary endpoint was the development of diabetes or death. After a median follow up time of three years, the incidence of this endpoint did not differ significantly between ramipril and placebo (18.1% vs. 19.5%; HR 0.91; 95% CI 0.81–1.03). Rosiglitazone reduced the endpoint significantly (n = F306; 11.6%) compared with placebo (n = 686; 26.0%; HR 0.40; 0.35–0.46; $P < 0.0001$). Thus the effect of rosiglitazone on the likelihood to develop diabetes in people with impaired glucose homeostasis was as could be expected considering its known glucose lowering property. Overall, total cardiovascular events did not differ significantly between the rosiglitazone and placebo groups. In the rosiglitazone group, however, body weight increased significantly ($P < 0.0001$) and more heart failure cases (0.5 vs. 0.1%; $P < 0.01$) were found The DREAM trial was neither planned nor powered to evaluate cardiovascular outcomes, which would have demanded a longer trial period. Also, a longer follow-up is needed to see whether the glucometabolic effect of rosiglitazone on glucose only lasts as long as the treatment is continued, or if it is sustained. Thus, rosiglitazone cannot, until further evidence has been gained, be considered appropriate management to reduce the risk of cardiovascular disease in people with impaired glucose homeostasis.

The recent data from the STOP-NIDDM Trial have for the first time suggested that acute cardiovascular events in people with IGT may be prevented by treatment that reduces post-prandial glucose levels.[70] Furthermore, data based on NHANES III have shown that control of LDL-cholesterol, HDL-cholesterol, and blood pressure to normal levels in patients with the metabolic syndrome (without diabetes and CAD) would result in preventing 51% of coronary events in men and 43% in women; control of these risk factors to optimal levels would result in preventing 81% and 82% of events, respectively.[183]

The identification of high-risk subjects for type 2 diabetes is relatively easy; no biochemical or other costly tests are required. It is now recognized, that from a prevention point of view, screening for type 2 diabetes is not the same as measuring blood glucose, but one can determine the risk of developing type 2 diabetes using non-invasive data before testing for blood glucose. Diabetes risk scores have been developed in several populations.[31,184,185] It is also important to note that most of

Table 5 Summary of the findings of four life style intervention studies that aimed at preventing type 2 diabetes in subjects with IGT

Study	Cohort size	Mean BMI (kg/m^2)	Duration (years)	RRR (%)	ARR (%)	NNT
Malmö[174]	217	26.6	5	63	18	28
DPS[108]	523	31.0	3	58	12	22
DPP[109]	2161[a]	34.0	3	58	15	21
Da Qing[175]	500	25.8	6	46	27	25

RRR = relative risk reduction; ARR = absolute risk reduction/1000 person-years; NNT = numbers needed to treat to prevent one case of diabetes over 12 months.
[a]Combined numbers for placebo and diet and exercise groups.

the high-risk subjects for type 2 diabetes are already regular customers of primary health care services for various reasons. What is needed in the health care system is to determine the future risk of type 2 diabetes using easy tools such as the FINDRISC in individuals that seem to be potential candidates of type 2 diabetes. The individuals with high scores should be systematically targeted with life style intervention regardless of their current glucose levels. It is necessary that such an intervention will become a part of the routine preventive care in order to reduce the burden of type 2 diabetes.

Recommendation
People at high risk for type 2 diabetes, should receive appropriate life style counselling and if needed pharmacological therapy to reduce or delay their risk of developing diabetes. This may also decrease their risk of CVD. Class I, Level of Evidence A.

In people with IGT, the onset of diabetes can be delayed by certain drugs (such as metformin, acarbose and rosiglitazone). Class I, Level of Evidence A.

Prevention of CVD by physical activity

In previous years, several studies assessed the association between physical activity and the risk of cardiovascular mortality among diabetic patients.[186-191] The results of these studies indicated that regular leisure-time physical activity is associated with reduced CVD and total mortality among patients with diabetes. Walking had a similar inverse association with the risk of cardiovascular and total mortality as vigorous leisure-time physical activity.[186-189] In the Aerobic Center Longitudinal Study, the low fitness group had a high relative risk for total mortality compared with the fit group.[186] Other types of physical activity, such as occupational and daily commuting physical activity on foot or by bicycle, have also been found to be associated with reduced cardiovascular mortality among diabetic patients;[191] people physically active at their work had a 40% lower cardiovascular mortality compared with people with low physical activity at work. A high level of leisure-time physical activity was associated with a 33% drop in cardiovascular mortality and moderate activity was linked to a 17% drop in cardiovascular mortality compared to the most sedentary group. Daily walking or cycling to and from work decreased cardiovascular mortality, but this relation was no longer significant after additional adjustment for occupational and leisure-time physical activity. Simultaneously doing one, two, or three types of moderate or high occupational, commuting, and leisure-time physical activity reduced significantly total and CVD mortality.[191] Thus, data from the observational studies suggest that the reduction in cardiovascular risk associated with physical activity may be comparable with that of pharmacological treatment prescribed to patients with type 2 diabetes. The ADA, the National Cholesterol Education Programme Expert Panel, and International Diabetes Federation (European Region) have recommended physical activity for the primary and secondary prevention of CVD complications among diabetic patients.[127,193,194] The assessment of the level of physical activity is not difficult. It can be done using simple questionnaires, or using pedometers, or more sophisticated equipments. The most important thing is that it is done and that health workers motivate diabetic patients to be physically active.

Recommendation
Diabetic patients should be advised to be physically active in order to decrease their cardiovascular risk. Class I, Level of Evidence A.

Pathophysiology

Role of hyperglycaemia

Hyperglycaemia characterizes both type 1 and type 2 DM. Since a number of studies closely linked elevated blood glucose levels to excess mortality and morbidity from vascular disease,[64] growing efforts currently focus on clarifying the effects of glucose on vascular function in particular endothelial function and nitric oxide (NO) bioavailability.

NO causes vasodilation and platelet inhibition and thereby prevents vasoconstriction and thrombus formation. NO is generated by a family of enzymes called NO synthase[195,196] One of these enzymes, i.e. endothelial NOS (eNOS), is Ca^{2+}-dependent and constitutively present in various types of cells, including endothelial cells. The activity of the L-arginine/NO pathway is a balance between synthesis and breakdown of NO by its reaction with the superoxide anion (O_2^-). Under physiological conditions, the production of this molecule is not markedly affected by O_2^-. Hence, NO may exert its well-known vascular protective effects favouring an anti-atherosclerotic environment. However, in the presence of cardiovascular risk factors, an excessive production of O_2^- occurs rapidly inactivating NO and leading to the formation of high concentrations of peroxynitrite ($ONOO^-$), a very powerful oxidant (*Figure 9*).

Several lines of evidence support the concept that hyperglycaemia decreases endothelium-derived NO availability and affects vascular function[198,199] *via* a number of mechanisms as depicted in *Figure 10*, mainly involving overproduction of reactive oxygen species (ROS), namely O_2^-.[200]

The mitochondrial electron transport chain is probably one of the first targets of high glucose, with a direct net increase in O_2^- formation. A further increase in O_2^- production is driven by a vicious circle involving ROS-induced activation of protein kinase C (PKC) and *vice versa* (*Figure 11*). Indeed, activation of PKC by glucose has been implicated in the regulation and activation of membrane-associated $NAD(P)H^-$-dependent oxidase, this latter leading to subsequent production of superoxide anion.[202] Indeed, NAD(P)H activity and subunit protein expression are enhanced in internal mammary arteries and

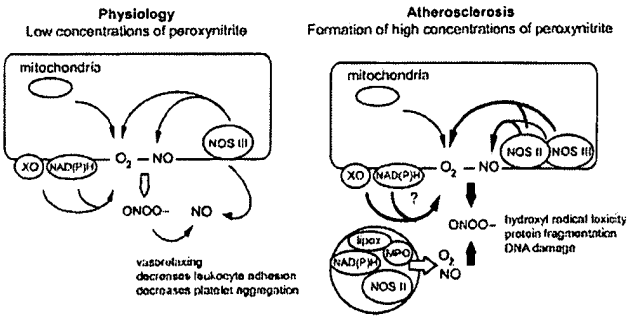


Figure 9 In the atherosclerotic setting, an excessive production of O_2^- occurs. O_2^- rapidly inactivates NO leading to the formation of high concentrations of peroxynitrite ($ONOO^-$), a condition associated with cellular toxicity. Please note the putative sources of O_2^- in the left panel.[197]

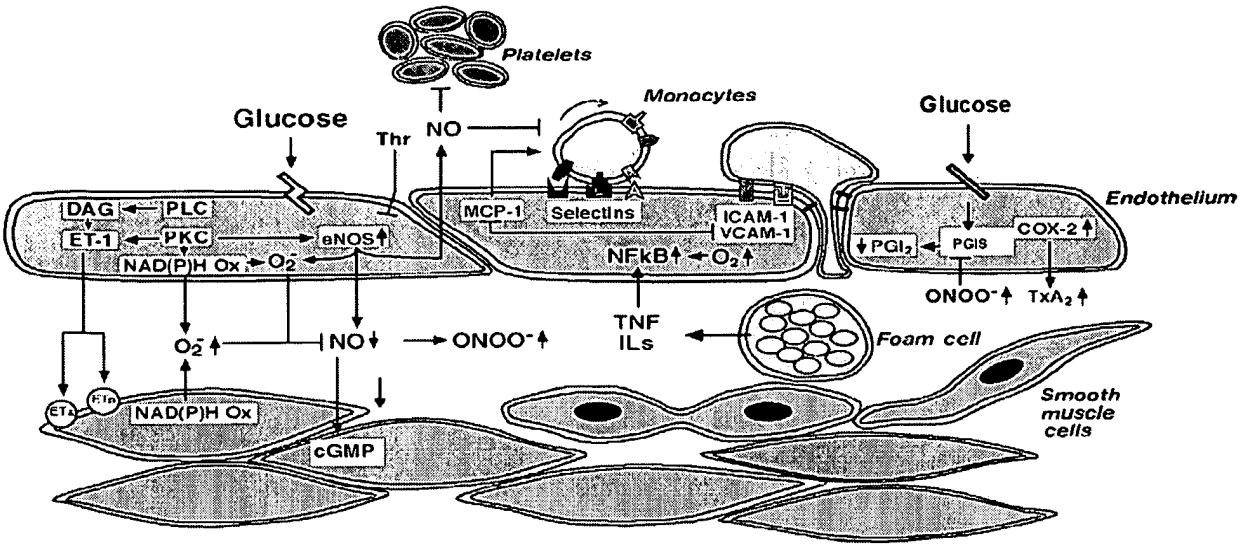


Figure 10 Hyperglycaemia and endothelium-derived vasoactive substances. DAG, diacylglycerol; PKC, protein kinase C; PLC, phospholipase C; eNOS, endothelial nitric oxide synthase; Thr, thrombin; NADPH Ox, nicotinamide adenine dinucleotide phosphate oxidase; O_2^-, superoxide anion; $ONOO^-$, peroxynitrite; MCP-1, monocyte chemoattractant; TNF, tumour necrosis factor; ILs, interleukins; COX-2, cyclooxygenase-2.[201]

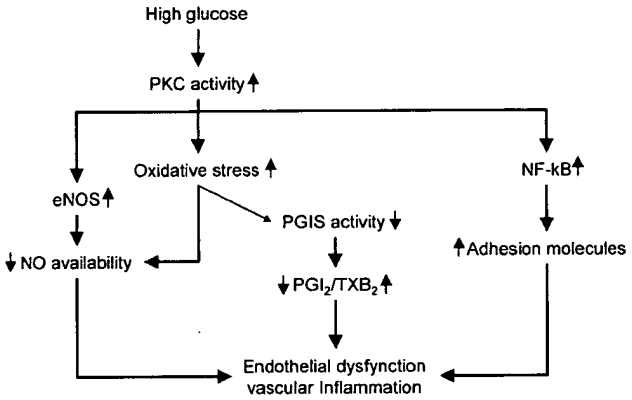


Figure 11 A single unifying PKC-dependent mechanism is the triggering step by which hyperglycaemia induces endothelial dysfunction and vascular inflammation. PKC, protein kinase C; PGIS, prostacyclin synthase; PGI_2/TXB_2, prostacyclin/thromboxane.

saphenous veins of diabetic patients.[203] Moreover, high glucose-dependent PKC activation induces an upregulation of inducible COX-2 and eNOS expression as well as a selective increase of thromboxane production and reduced NO release. Hence, activation of the PKC pathway represents a proximal node in the intracellular signalling leading to hyperglycaemia-induced oxidative stress and endothelial dysfunction.[204]

Oxygen-derived free radical excess affects endothelial function *via* a number of different pathways: (i) O_2^- rapidly inactivates NO to peroxynitrite,[205] a powerful oxidant which easily penetrates across phospholipid membranes and produces substrate nitration, thereby inactivating regulatory receptors and enzymes, such as free radical scavengers[206,207] and key NOS co-factors, for instance tetrahydrobiopterin;[208] (ii) mitochondrial production of superoxide increases intracellular formation of advanced glycation end-products (AGEs), which adversely affect endothelial function by increasing ROS production and inflammatory cytokines from vascular cells, thereby enhancing endothelial expression of various adhesion molecules implicated in atherogenesis;[209] (iii) activation of the receptor for AGEs (RAGE) increases intracellular superoxide anion production[210] and seems to represent a key step in atherosclerotic lesion development;[211] (iv) O_2^- production activates the hexosamine pathway, which lowers Akt-induced NOS activation.[212] Akt activation is further limited by PKC-dependent inhibition of phosphatidylinositol-3 kinase pathway; (v) high glucose-induced oxidative stress increases the levels of dimethylarginine, a competitive antagonist of NOS.[213]

The impact of DM on vascular function is not limited to the endothelium. In patients with type 2 DM, the vasodilator response to exogenous NO donors is diminished.[214] Dysregulation of vascular smooth muscle function is further enhanced by impairments in sympathetic nervous system function. Diabetes increases protein kinase C (PKC) activity, NF-kB production, and generation of oxygen-derived free radicals in vascular smooth muscle, akin to these effects in endothelial cells. Moreover, diabetes heightens migration of vascular smooth muscle cells into nascent atherosclerotic lesions, where they replicate and produce extracellular matrix—important steps in mature lesion formation.[215] Vascular smooth muscle cell apoptosis in atherosclerotic lesions is also increased, such that patients with diabetes tend to have fewer smooth muscle cells in the lesions, which increases the propensity for plaque rupture.[216] In persons with diabetes, production of cytokines diminishes vascular smooth muscle synthesis of collagen and increases production of matrix metalloproteinases, resulting in an increased tendency for plaque destabilization and rupture.

Given the above effects of hyperglycaemia on vascular function, one might speculate that tight glycaemic control warrants preservation from micro- and macrovascular damage and that it favourably impacts prognosis in diabetic patients. Epidemiological studies support the notion that increasing blood glucose levels proportionally relates to

cardiovascular events. Less is known on the effect of very strict glycaemic control. In the United Kingdom Prospective Diabetes Study (UKPDS),[71] the risk of death, stroke, or amputation did not change while there was a trend towards less MIs in the most actively treated group. Glycaemic control was, however, rather modest with a glycated haemoglobin A_{1c} (HbA_{1c}) of 7% in the intervention group and only a small difference of 0.9% between the intervention and the control groups. Still, improved treatment of hyperglycaemia lowered the incidence of diabetic retinopathy and nephropathy.

Role of insulin resistance and β-cell dysfunction

Insulin resistance is a typical characteristic of type 2 diabetes. Insulin stimulates NO production from endothelial cells by increasing the activity of NOS via activation of phosphatidylinositol-3 kinase (PI-3K) and Akt kinase. Thus, in healthy subjects, insulin increases endothelium-dependent (NO-mediated) vasodilatation. On the contrary, endothelium-dependent vasodilatation is reduced in insulin-resistant subjects. Furthermore, insulin-mediated glucose disposal correlates inversely with the severity of the impairment in endothelium-dependent vasodilatation. Abnormal endothelium-dependent vasodilatation in insulin-resistant states may be explained by alterations in intracellular signalling that reduce the production of NO. Insulin signal transduction via the PI-3K pathway is impaired and insulin is less able to produce NO. On the other hand, insulin signal via the mitogen-activated protein kinase pathway (MAPK) remains intact. MAPK activation is associated with increased endothelin production and a greater level of inflammation and thrombosis. Insulin resistance is a distinct trait of DM, and its magnitude directly relates to cardiovascular outcomes.[217,218]

Evidence has accumulated during the last decade on the significant roles of both decreased insulin sensitivity and β-cell dysfunction in the pathogenesis of type 2 DM. The relative importance and causal relations of these disturbances in the pathogenesis of diabetes are under debate. Both insulin secretion and insulin sensitivity are genetically and environmentally controlled and the impairment of both has individually or together been associated with increasing risk of developing type 2 DM.[219-221] It has been reported that during each stage of the development of type 2 DM, decreased insulin sensitivity and insulin secretory dysfunction are independent predictors of worsening glucose tolerance.[222] Indeed, fasting hyperinsulinaemia, known to reflect decreased insulin sensitivity, together with decreased insulin secretion are the strongest independent predictors of type 2 DM.[223] Furthermore, decreased β-cell function may exist already at normal FPG levels.[224] Several studies have presented results that indicate that β-cell dysfunction in early stages of abnormal glucose tolerance is independent of insulin resistance and already present in obese patients with normal glucose tolerance.[225-227] In addition, a study of type 2 diabetic Japanese patients recently showed that decreased insulin secretion had a more pronounced impact on glucose tolerance than insulin sensitivity.[228] Both glucose toxicity and lipotoxicity have been reported to contribute to β-cell dysfunction. The lipotoxicity has above all been associated with elevated plasma free fatty acids (FFA), which have been observed to hamper insulin secretion through toxic effects on the β-cells.[229,230] Furthermore, long-term lowering of plasma FFA concentrations improves the acute insulin response and insulin-mediated glucose uptake.[231] A recent study[232] has shown that glucose abnormalities in patients with acute MI (AMI), but without previously known type 2 DM to a significant extent are related to impaired β-cell secretion of insulin. This confirms that hyperglycaemia immediately after an infarction is not a stress-epiphenomenon but reflects stable disturbances of glucose regulation preceding the AMI. Early β-cell dysfunction may have important pathophysiological implications and may serve as a future treatment target.

Risk factors for atherosclerosis

Oxidative stress

Given the pivotal role of oxidative stress on endothelial function and atherosclerotic processes in diabetes, growing efforts focus on the putative effects of antioxidant therapy. Despite evidence indicating reversal of endothelial dysfunction by different antioxidant agents,[233] data from clinical trials are still inconclusive and presently it does not support an indication for antioxidant therapy in DM.[234,235] These data would seem to refute a role of oxidative stress in the pathogenesis of atherosclerosis. There are several reasons to believe that this conclusion is not justified but rather that treatment with antioxidants is perhaps not the best approach for reducing oxidant stress. First, the rate of the reaction between vitamin E and superoxide is several orders of magnitude less than the rate of the reaction of superoxide with NO. Secondly, many of the oxidative events occur in the cytoplasm and in the extracellular space, and would not be affected by lipid-soluble antioxidants that are concentrated in lipid membranes and lipoproteins. Thirdly, antioxidants may become pro-oxidants after scavenging a radical, vitamin E and C become tocopheroxyl and ascorbyl radicals, respectively. The tocopheroxyl radical can be regenerated by other antioxidants such as vitamin C or co-enzyme Q10. For this reason, the use of cocktails of antioxidants rather than high doses of a selected one may be more effective. Given the above considerations, it is quite possible that use of antioxidant vitamins will never prove to be the best approach to limit vascular oxidant stress. To prevent the development of the earliest stages of diabetic vascular disease, future research should focus on identifying substances which have antioxidant effects not because they scavenge radicals, but because they block the production of radicals.

Lipid disorders

Classically, DM induces elevation in triglyceride and LDL, and decline in HDL plasma levels. These changes clearly affect the natural history of the atherosclerotic disease, and render patients with diabetes more prone to develop CAD, stroke, and peripheral vascular disease. Recent evidence confers to diabetes-related enhanced FFA liberation, a crucial role in producing the well-described changes in lipid profile. Excess circulating levels of FFA results from both enhanced release from adipose tissue and reduced uptake by skeletal muscle.[236] The liver responds to FFA excess by increasing VLDL production and cholesteryl ester synthesis.[237] The accumulation of triglyceride-rich

lipoproteins, depends also on their reduced clearance by lipoprotein lipase, triggers hypertriglyceridaemia and lowers HDL levels by promoting exchanges from HDL to VLDL *via* cholesteryl ester transfer protein.[237] HDLs are not only reduced in quantity, but also impaired in function. Indeed, HDL from poorly controlled type 2 diabetic patients are less effective in preventing LDL oxidation compared to those from non-diabetic subjects.[238] Moreover, increased VLDL production and abnormal cholesterol and triglyceride transfer between VLDL and LDL enhances plasma levels of small and dense proatherogenic LDLs,[239] which are in addition more prone to oxidation due to impaired antioxidant defence mechanisms in the plasma of diabetics.[240] The proatherosclerotic effects of these particles on coronary, carotid, and peripheral arteries, have important clinical consequences, thus representing an important treatment target.

Thrombosis and coagulation

Platelet function is crucial in determining the natural history of atherosclerosis and consequences of plaque rupture. It is therefore not surprising that cardiovascular risk is closely linked to platelet function abnormalities and coagulation disorders in the diabetic patient. The intracellular platelet glucose concentration mirrors the extracellular environment and is associated with increased superoxide anion formation, PKC activity, and decreased platelet-derived NO.[241,242] Moreover, diabetic patients show increased expression of glycoprotein Ib and IIb/IIIa, which enhances both platelet-von Willebrand factor and platelet-fibrin interaction. Hyperglycaemia further affects platelet function by impairing calcium homeostasis,[243] and thereby altering platelet conformation, secretion and aggregation, and thromboxane formation. Further abnormalities affecting platelet function include impaired endothelial production of nitric oxide and prostacyclin, and increased production of fibrinogen, thrombin, and von Willebrand factor. Moreover, blood coagulability is enhanced in diabetic patients. Indeed, plasma coagulation factors (e.g. factor VII and thrombin), lesion-based coagulants (e.g. tissue factor), plasminogen activator inhibitor-1 (PAI-1) (a fibrinolysis inhibitor) are increased, and endogenous anticoagulants (e.g. thrombomodulin and protein C) are decreased.[244-248] Thus, a propensity for platelet activation and aggregation, coupled with a tendency for coagulation, amplify the risk that plaque rupture result in thrombotic occlusion of arteries (*Figure 12*).

Non-atherosclerotic CVD

Diabetic cardiomyopathy

Metabolic perturbations in the myocardial cell are the most probable causes for myocardial dysfunction in patients with diabetes. The dominant pathway for myocardial energy production is beta-oxidation of FFAs, but the myocardium is also to a lesser extent dependent on glucose oxidation (*Figure 13*).

When the heart is subjected to ischaemic stress or exposed to sustained enhancement of intraventricular pressure, its ATP production changes towards a more dominant glucose oxidation.[249] In diabetes, glucose for energy production is, however, substantially lower, accounting for only about 10% of the myocardial energy production. The shift to a more pronounced beta-oxidation of FFA causes therefore a higher oxygen utilization than under normal circumstances.[250] Thus, in diabetes and heart failure, the heart is exposed to increased concentrations of FFAs, released via stress influenced by an increased sympathetic tone as well as through insulin resistance and insulin deficiency-enhancing lipolysis.[251,252] It has been proposed that prolonged intracellular accumulation of FFA and its metabolites may cause myocardial dysfunction.[253] Diabetic patients are also known to have increased risk for other disturbances such as reduced myocardial blood flow and blunted hyperkinetic response to myocardial ischaemia

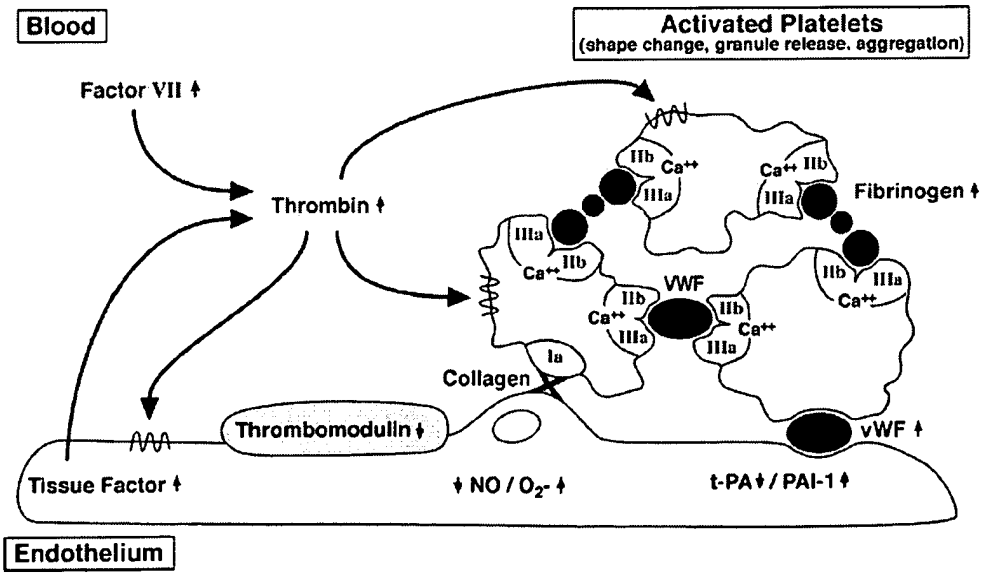


Figure 12 Platelet function and plasma coagulation factors are altered in diabetes, favouring platelet aggregation and a propensity for thrombosis. There is increased expression of glycoprotein Ib and IIb/IIIa, augmenting both platelet-von Willebrand (vWF) factor and platelet-fibrin interaction. The bioavailability of NO is decreased. Coagulation factors such as tissue factor, factor VII, and thrombin are increased; PAI-1 is increased; and endogenous anticoagulants such as thrombomodulin and tissue plasminogen activator (tPA) are decreased.[201]

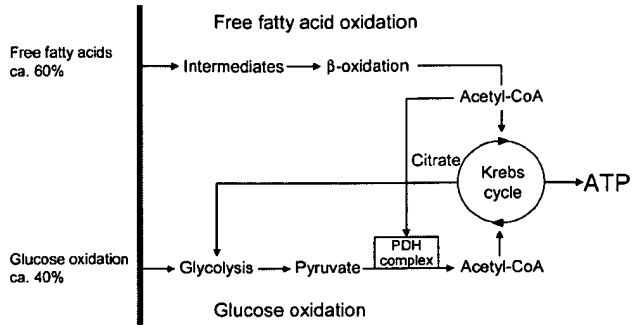


Figure 13 The majority of the ATP production in the myocardial cell is through beta oxidation of FFAs in normal hearts with a less contribution from glucose oxidation. This balance is affected by even more production through beta oxidation of FFAs in diabetes patients.

resulting in diminished myocardial function.[76,198,254,257] Recent evidence indicates that in the heart of type 2 diabetic patients, there is a mismatch between insulin-mediated glucose uptake and blood flow[162] in addition to the insulin resistance of myocardial muscle.[176]

Cardiac autonomic neuropathy

Autonomic neuropathy is a serious and common complication of diabetes. It has been estimated that about 20% of asymptomatic diabetic patients have abnormal cardiovascular autonomic function.[256,257] The risk for cardiovascular autonomic neuropathy depends on the duration of diabetes and the degree of glycaemic control. It is caused by injury to the autonomic nerve fibres that innervate the heart and blood vessels. The hypotheses concerning the aetiology of cardiovascular autonomic neuropathy include metabolic insult to nerve fibres, neurovascular insufficiency, neurohormonal growth factor deficiency, and autoimmune damage.[258] Main consequences are dysfunctional heart rate control, abnormal vascular dynamics, and cardiac denervation, which become clinically overt as exercise intolerance,[259] orthostatic hypotension,[260] intraoperative cardiovascular lability,[260] and silent myocardial ischaemia.

The earliest sign is often a vagal deficiency leaving sympathetic innervation unopposed. A manifestation of this is that diabetic patients tend to have higher resting heart rate and less heart rate variability during the day than their non-diabetic counterparts. A clinical setting where this may be particularly unfavourable is at the onset of an MI causing unnecessary myocardial oxygen consumption in a situation with decreased nutritional blood supply.

The autonomic nervous system influences coronary blood flow regulation independently of endothelial cell function. Diabetic patients with sympathetic nervous system dysfunction have impaired dilatation of coronary resistance vessels in response to cold pressure testing when compared with diabetics without defects in cardiac adrenergic nerve density. Global myocardial blood flow and coronary flow reserve studied by positron emission tomography in response to adenosine provocation were subnormal in diabetics with cardiovascular autonomic neuropathy. It is obvious that cardiovascular autonomic neuropathy may provoke ischaemic episodes by upsetting the balance between myocardial supply and demand. Due to autonomic neuropathy, silent myocardial ischaemia is prevalent in diabetic patients and often symptomatically apparent only in advanced stages of the disease. Instead of typical angina, patients often complain of shortness of breath, diaphoresis, or profound fatigue.

Knowledge on the actual prevalence of cardiovascular autonomic neuropathy and cardiovascular autonomic neuropathy-related mortality rates are conflicting. However, different studies and meta-analysis reveal that mortality rates among diabetic subjects with cardiovascular autonomic neuropathy are many times higher than among those without. Subjects with diabetes and low levels of autonomic function parameters (baroreflex sensitivity, heart rate variability, and classical Ewing tests) had an approximately doubled risk of mortality in the Hoorn Study.[262] In the Detection of Ischaemia in Asymptomatic Diabetics Study,[263] cardiac autonomic dysfunction, assessed by the Valsalva manoeuvre, was a strong predictor of ischaemia, whereas traditional and emerging risk factors were not. Impaired angina perception largely accounts for such an increased mortality. Indeed, silent myocardial ischaemia delays treatment of acute coronary events and makes it more difficult to monitor anti-ischaemic treatment or determine whether restenosis has occurred after a coronary intervention. Although silent, myocardial ischaemia has a reported prevalence of 10–20% in diabetic vs. 1–4% in non-diabetic populations, routine screening for silent myocardial ischaemia in diabetics remains debatable. In the Detection of Ischaemia in Asymptomatic Diabetics Study, 22% of 522 type 2 diabetic patients randomized to adenosine stress testing with myocardial perfusion imaging by means of SPECT had silent ischaemia. This would indicate that asymptomatic diabetic patients have at least an intermediate probability of CAD, a prevalence that has been considered as justifying routine screening for CAD by non-invasive testing. In a series of 203 diabetic patients,[264] the prevalence of functional silent myocardial ischaemia, assessed by stress ECG and thallium myocardial scintigraphy, was 15.7%. In this study, the positive predictive value of exercise ECG was 90%, compared with 63% of thallium myocardial scintigraphy. Thus, available evidence highlights the need for non-invasive screening by means of stress-testing in diabetic subjects, especially considering the high sensitivity, feasibility, and low costs of exercise ECG.

Based on cardiovascular autonomic neuropathy-associated coronary blood flow impairment, misdiagnosed CAD, and the consequently higher risk of mortality, it is presently recommended that a baseline determination of cardiovascular autonomic function is performed upon diagnosis in type 2 diabetes and within 5 years of diagnosis for type 1 diabetes, followed by yearly repeated tests.[265]

Metabolic syndrome

In 1988, Reaven[118] noted that several risk factors (e.g. dyslipidaemia, hypertension, hyperglycaemia) commonly cluster together. He defined this clustering as Syndrome X, and identified it as a risk factor for CVD. Presently, there is an ongoing debate whether this clustering of cardiovascular risk factors represents a disease entity in its own right.[130,266,267] However, in clinical practice it helps identifying individuals at high-risk for CVD and type 2 diabetes. In the following, the concept of the metabolic syndrome is reviewed.

Table 6 Criteria for the metabolic syndrome according to ATP III[269]

Risk factor	Defining level
Abdominal obesity, given as waist circumference[a,b]	
Men	>102 cm (>40 in.)
Women	>88 cm (>35 in.)
Triglycerides	≥1.7 mmol/L (≥150 mg/dL)
HDL cholesterol	
Men	<1.03 mmol/L (<40 mg/dL)
Women	<1.29 mmol/L (<50 mg/dL)
Blood pressure	≥130/≥85 mm Hg
Fasting glucose	≥6.1 mmol/L (≥110 mg/dL)[c]

[a]Overweight and obesity are associated with insulin resistance and the metabolic syndrome. However, the presence of abdominal obesity is more highly correlated with the metabolic risk factors than is an elevated BMI. Therefore, the simple measure of waist circumference is recommended to identify the body weight component of the metabolic syndrome.

[b]Some male patients can develop multiple metabolic risk factors when the waist circumference is only marginally increased, e.g. 94–102 cm (37–39 in.). Such patients may have a strong genetic contribution to insulin resistance. They should benefit from changes in life habits, similarly to men with categorical increases in waist circumference.

[c]The ADA has recently established a cut-point of ≥100 mg/dL, above which persons have either pre-diabetes (IFG) or diabetes. This new cut-point should be applicable for identifying the lower boundary to define an elevated glucose as one criterion for the metabolic syndrome.

Table 7 Criteria for metabolic syndrome according to WHO[269]

Insulin resistance, identified by one of the following
Type 2 diabetes
Impaired fasting glucose
IGT
Or for those with normal fasting glucose levels <6.1 mmol/L (<110 mg/dL), glucose uptake below the lowest quartile for background population under investigation under hyperinsulinaemic, euglycaemic conditions
Plus any two of the following
Anti-hypertensive medication and/or high blood pressure (≥140 mm Hg systolic or ≥90 mm Hg diastolic)
Plasma triglycerides ≥1.7 mmol/L (≥150 mg/dL) and/or HDL cholesterol <0.9 mmol/L (<35 mg/dL) in men or <1.0 mmol/L (<39 mg/dL) in women
BMI >30 kg/m^2 and/or waist-to-hip ratio >0.9 in men, >0.85 in women
Urinary albumin excretion rate ≥20 μg/min or albumin:creatinine ratio ≥30 mg/g

Table 8 Criteria for metabolic syndrome according to International Diabetes Federation[130]

Central obesity (defined as waist circumference ≥94 cm for Europid men and ≥80 cm for Europid women, with ethnicity specific values for other groups)
Plus any two of the following four factors
Raised TG level: ≥1.7 mmol/L (150 mg/dL), or specific treatment for this lipid abnormality
Reduced HDL cholesterol: <1.03 mmol/L (40 mg/dL) in males and <1.29 mmol/L (50 mg/dL) in females, or specific treatment for this lipid abnormality
Raised blood pressure: systolic BP ≥ 130 or diastolic BP ≥ 85 mm Hg, or treatment of previously diagnosed hypertension
Raised FPG ≥5.6 mmol/L (100 mg/dL), or previously diagnosed type 2 diabetes
If above 5.6 mmol/L or 100 mg/dL, OGTT is strongly recommended, but is not necessary to define presence of the syndrome

Table 9 Ethnic-specific values for waist circumference[130]

Country/ethnic group		Waist circumference[a]
Europids	Male	≥94 cm
In the USA, the ATP III values (102 cm male; 88 cm female) are likely to continue to be used for clinical purposes	Female	≥80 cm
South Asians	Male	≥90 cm
Based on a Chinese, Malay, and Asian–Indian population	Female	≥80 cm
Chinese	Male	≥90 cm
	Female	≥80 cm
Japanese	Male	≥85 cm
	Female	≥90 cm
Ethnic South and Central Americans	Use South Asian recommendations until more specific data are available	
Sub-Saharan Africans	Use European data until more specific data are available	
Eastern Mediterranean and Middle East (Arab) populations	Use European data until more specific data are available	

[a]In future, epidemiological studies of populations of Europid origin, prevalence should be given using both European and North American thresholds to allow better comparisons.

Nowadays, extended panels of metabolic risk factors have been identified in order to better understand pathogenesis, predict outcomes, and improve clinical management of the so-called metabolic syndrome. Two independent efforts to identify definition criteria have been carried out by the National Cholesterol Education Programme's Adult Treatment Panel III (ATP III)[126] and the World Health Organization (WHO).[122]

ATP III identified six components of the metabolic syndrome that relate to CVD: (i) abdominal obesity; (ii) atherogenic dyslipidaemia; (iii) raised blood pressure; (iv) insulin resistance and/or glucose intolerance; (v) pro-inflammatory state; and (vi) prothrombotic state.

All of these components are part of a larger body of risk factors for CVD that ATP III identifies as *underlying* (obesity, physical inactivity, atherogenic diet), *major* (cigarette smoking, hypertension, elevated LDL cholesterol, low HDL cholesterol, family history of premature CHD, aging), and *emerging* (elevated triglycerides, small LDL particles, insulin resistance, glucose intolerance, pro-inflammatory state, and prothrombotic state).

In order to facilitate diagnosis and preventive interventions, ATP III proposed a clinical definition based on having at least three of five criteria (*Table 6*). Using ATP III definition, the estimated prevalence of the metabolic syndrome among men and women in NHANES III[270] ranges from 5%

(normal weight) to 60% (obese) in men, and from 6% (normal weight) and 50% (obese) in women. Currently, it exceeds 20% of individuals who are at least 20 years of age, and 40% of the population >40 years.[271]

The WHO criteria (*Table 7*) require insulin resistance for diagnosis, by demonstrating the presence of type 2 diabetes, IFG, or IGT by OGTT in patients without IFG. In addition to insulin resistance, two other risk factors are sufficient for the diagnosis of metabolic syndrome.

On the contrary, ATP III claims that information obtained from OGTT does not outweigh the inconveniences and costs by applying this test in clinical practice.

Notably, both ATP III and WHO recognize CVD as the primary outcome of the metabolic syndrome. In the Framingham Study, the metabolic syndrome alone predicted 25% of all new-onset CVD. In the absence of diabetes, the metabolic syndrome generally did not raise the 10-year risk for CAD to >20% (the threshold for ATP IIIs CAD risk equivalent). Notably, the 10-year cardiovascular risk in men with metabolic syndrome ranged from 10–20%, whereas it did not exceed 10% in most women, who also displayed a lower rate of CAD events during the 8-year follow-up.

In April 2005, a group of experts invited by the International Diabetes Federation (IDF) released a unified definition for the metabolic syndrome seeking worldwide acceptance and potential application.[130]

To be defined as having the metabolic syndrome according to the new definition, a person must present as outlined in *Tables 8* and *9*.

While the pathogenesis of the metabolic syndrome and each of its components is complex and not well understood, central obesity and insulin resistance are acknowledged as important causative factors. Abdominal circumference, a *sine qua non* condition for diagnosis of the syndrome, is the clinical screening factor for the metabolic syndrome. Abdominal obesity is much more associated with metabolic risk factors than BMI. The IDF consensus group has highlighted a number of other parameters that appear to be related to the metabolic syndrome which should be included in research studies to help determine the predictive power of these extra criteria for CVD and/or diabetes. As outlined earlier, none of the definitions of the metabolic syndrome has been validated prospectively.

Obesity

Abdominal obesity, as detected by increased waist circumference, is the first criterion listed. Its inclusion reflects the pivotal role assigned to abdominal obesity as a contributor to metabolic syndrome: obesity contributes to hypertension, high serum cholesterol, low HDL cholesterol and hyperglycaemia, and it associates with higher CVD risk. Excess visceral adipose tissue releases several products that apparently exacerbate these risk factors including FFAs, which overloads muscular and liver tissues with lipid, thus enhancing insulin resistance; PAI-1, which contributes to a prothrombotic state; CRP, which may signify cytokine excess and a pro-inflammatory state. The strong connection between abdominal obesity and risk factors led ATP III to define the metabolic syndrome essentially as a cluster of metabolic complications of obesity.

Insulin resistance

Insulin resistance *per se* is believed to play a significant role in the pathogenesis of the metabolic syndrome, and many investigators claim that insulin resistance is the pathophysiological process behind the clustering of cardiovascular risk factors in the metabolic syndrome.[272] Insulin resistance predicts atherosclerosis and cardiovascular events independently of other risk factors, including fasting glucose and lipid levels.[273] However, so far there is little evidence that a reduction in insulin resistance will substantially improve any of the components of the metabolic syndrome other than glucose intolerance. Moreover, identifying a unique role for insulin resistance is complicated by the fact that it is often linked to obesity. Thus, the putative mechanistic link between insulin resistance and most of the components of the metabolic syndrome remains unclear and does not meet general consensus. Mechanisms by which insulin resistance impacts other metabolic syndrome risk factors are: (i) diversion of excess FFAs from lipid-overloaded insulin-resistant muscles to the liver, thus promoting fatty liver and atherogenic dyslipidaemia; (ii) enhanced output of VLDL; (iii) predisposition to glucose intolerance, which can be worsened by increased hepatic gluconeogenesis in the insulin-resistant liver; (iv) increasing blood pressure.

Insulin resistance generally rises with increasing body fat and most people with a BMI $\geq 30\,kg/m^2$ have postprandial hyperinsulinaemia/reduced insulin sensitivity,[274] whereas persons with a BMI of 25–29.9 kg/m^2 exhibit a spectrum of insulin sensitivities as well. In some populations (such as South Asians), insulin resistance is common even with BMI $<25\,kg/m^2$, a condition termed primary insulin resistance.

To reduce insulin resistance is an attractive pharmacological target to prevent CVD in metabolic syndrome patients currently with two classes of drugs available: metformin and insulin sensitizers, such as thiazolidinediones (TZDs). Both drugs reduce insulin resistance and favourably impact different metabolic risk factors. However, no clinical trial documented the efficacy of metformin and TZDs in reducing CVD risk, thus limiting recommendation for their use in preventing CVD in patients with either the metabolic syndrome or diabetes. Anyhow, good glycaemic control obtained through drug therapy and life style changes is mandatory.

Atherogenic dyslipidaemia

Atherogenic dyslipidaemia is often recognized in the metabolic syndrome and manifests itself by raised triglycerides, low HDL cholesterol, increased remnant lipoproteins, elevated apolipoprotein B, small LDL, and small HDL particles. It is commonly believed that hypertriglyceridaemia is due to enhanced triglyceride hepatic synthesis driven by an increased flux of FFAs from the periphery to the liver in an insulin-resistant setting.[275] The causes of hypertriglyceridaemia in the metabolic syndrome are, however, most likely multifactorial and the increased FFA flux-hypothesis is only one side of this issue. In the same view, low HDL cholesterol, often ascribed to elevated TG because of increased transfer of TG to HDL and cholesterol from HDL,[276] are likely to have a more complex origin, since HDL cholesterol levels are often reduced in patients with insulin resistance even when fasting TG levels are normal.

More recently, activation of innate immunity and immunity-related inflammation have been proposed as potential links between insulin resistance and dyslipidaemia. In animal models activation of innate immunity leads to changes in lipoproteins, enzymes, transfer proteins, and receptors[277] commonly seen also in human metabolic syndrome. Inflammation-driven increase in lipase production has been proposed as a mechanism promoting reduction of lipid content of HDLs.[278]

High blood pressure

In obese patients, blood pressure is sensitive to sodium intake, and this sensitivity is related to fasting insulin levels.[279] The anti-natriuretic effect of insulin, together with its ability to activate the sympathetic nervous system[280] and to drive abnormal vascular function contributes to the development of hypertension. Moreover, both hyperglycaemia and insulin activate the renin–angiotensin system (RAS) by enhancing the expression of angiotensinogen, angiotensin II, and AT1 receptor, which contributes to an increase in blood pressure in patients with insulin resistance.

Proinflammatory and prothrombotic state

Chronic subclinical inflammation is part of the metabolic syndrome.[281] This condition is characterized by elevated cytokines (e.g. TNF-alpha and IL-6) and acute-phase reactants (C-reactive protein and fibrinogen). Of interest, recent studies suggest that immunity and inflammation play a role in the development of insulin resistance and predict the development of type 2 DM.[282,283] Thus, the pathogenesis of insulin resistance and metabolic syndrome risk factors may have a common inflammatory basis, which closely relates to the occurrence of atherosclerotic cardiovascular events. Since measures of inflammatory activity do not presently provide additional insights into the risk of events in metabolic syndrome patients, the current clinical approach to the metabolic syndrome does not incorporate measurement of inflammatory markers. However, as elevated C-reactive protein levels ($\geq$3 mg/L) have been outlined as an emerging risk factor for CVD, its inclusion together with traditional metabolic syndrome risk factors into a single algorithm is likely to provide a useful approach to risk prediction in metabolic syndrome patients. Indeed, in a recently published study,[284] plasma C-reactive protein levels provided prognostic information regarding the risk of cardiovascular events in apparently healthy women at all levels of severity of the metabolic syndrome.

A prothrombotic state in patients with the metabolic syndrome is characterized by elevations of fibrinogen, PAI-1, and possibly other coagulation factors. In the metabolic syndrome, activation of NF-kB promotes synthesis of PAI-1, a natural inhibitor of tissue plasminogen activator, and leads to impaired fibrinolysis. PAI-1 levels correlate with plasma insulin levels and insulin resistance, and appear to predict the likelihood of developing diabetes.[285] Since no drugs are available that target PAI-1 and fibrinogen, the alternative approach to the prothrombotic state is antiplatelet therapy.

Treatment to reduce cardiovascular risk

Table of Recommendations:

Recommendation	Class[a]	Level[b]
Structured patient education improves metabolic and blood pressure control	I	A
Non-pharmacological life style therapy improves metabolic control	I	A
Self-monitoring improves glycaemic control	I	A
Near normoglycaemic control (HbA1c = 6.5%)[c]		
Reduces microvascular complications	I	A
Reduces macrovascular complications	I	A
Intensified insulin therapy in type 1 diabetes reduces morbidity and mortality	I	A
Early escalation of therapy towards predefined treatment targets improves a composite of morbidity and mortality in type 2 diabetes	IIa	B
Early initiation of insulin should be considered in patients with type 2 diabetes failing glucose target	IIb	C
Metformin is recommended as first line drug in overweight type 2 diabetes	IIa	B

[a]Class of recommendation.
[b]Level of evidence.
[c]DCCT-standardized.

Life style and comprehensive management

Long-term hyperglycaemia, i.e. DM–both type 1 and type 2–is strongly associated with specific microvascular complications of the retina and the kidneys on one side, and with abundant macrovascular disease of the heart, brain, and lower limbs as well as with neuropathy of the autonomic and peripheral nerve system on the other.[286–294] Macrovascular events are about 10 times more common than severe microvascular complications, and already occur at excessive rates in patients with glucometabolic disturbances, even before the onset of overt type 2 diabetes.[295–297] Hyperglycaemia is only one of a cluster of vascular risk factors which often is referred to as the metabolic syndrome.[118,131,135,300] Hence, treatment modalities have to be rather complex and strongly based on non-pharmacological therapy including life style changes and self-monitoring and it requires structured patient education.[301–305] This has to include a heavy emphasis on smoking cessation.

Implementation of a healthier life style with an increase in physical activity and a reduction of body weight, based on the regulation of calories and fat intake, are the basis for the prevention of type 2 diabetes,[108,109,306,307] and the same principles apply to basic treatment of type 2 diabetes.[301,302] Prior to treatment randomization, patients enrolled into the UKPDS, still to date the largest prospective treatment study in type 2 diabetes, underwent 3 months of non-pharmacological treatment with emphasis on life-style changes. Along with an average decrease of about 5 kg body weight, HbA_{1c} decreased about 2% to an

absolute value close to 7%.[303] Hence, non-pharmacological therapy seems to be at least as effective as any glucose-lowering drug therapy, which yields a mean HbA_{1c} lowering effect of 1.0–1.5% in placebo-controlled randomized studies (*Table 10*). Accordingly, non-pharmacological life style-oriented therapy is the foundation of all successful glucose-lowering regimens.

The specific recommendations include 30 min of physical activity at least five times a week, restriction of calorie intake to ~1500 kcal/day, restriction of fat intake to 30–35% of total daily energy uptake (reservation of 10% for monounsaturated fatty acids, e.g. olive oil), avoidance of *trans* fats, increased fibre uptake to 30 g/day, and the avoidance of liquid mono- and disaccharides.[108,109,301,303,307,308]

Risk stratification according to the coexistence of the metabolic syndrome and its individual features, or for concomitant-associated hypertension, dyslipidaemia, and microalbuminuria is mandatory for comprehensive management of patients with diabetes.[131,135,275,298,299,300] The recognition of the underlying insulin resistance with increased visceral adiposity is a key factor for an appropriate therapy, not only of hyperglycaemia but also of hypertension and dyslipidaemia.[269-300] The suggested new global definition of the metabolic syndrome, as recently released by the International Diabetes Federation, is depicted in *Table 6* (see section on Pathophysiology). Using this approach and applying multiple risk factor interventions to high-risk type 2 diabetic patients, as done in the Steno 2 Study, is extremely compelling in terms of overall outcome.[309] Targeting hyperglycaemia, hypertension, and dyslipidaemia together with the administration of acetylsalicylic acid to high-risk patients with established microalbuminuria resulted in a more than 50% reduction of major macrovascular events (fatal and non-fatal MI and stroke) with an NNT of as low as five over a 8-year long period ($P = 0.008$). This multiple risk factor intervention approach proved highly effective in less than 4 years in terms of microvascular outcomes, thereby confirming the results of UKPDS. Still, the ability to achieve pre-defined targets in Steno 2 was far from complete and strikingly variable. By far, the most difficult target to achieve was HbA_{1c} (*Figure 14*).

This notion was also apparent in the UKPDS,[71,291] fostering the concept of glucose-lowering polypharmacy, like antihypertensive therapy (see below). To reach targets is the crucial objective of comprehensive management. In this context and, in addition, every diabetic patient with some indication of vascular damage–be it macrovascular or microvascular–should be considered for antiplatelet drug therapy, especially acetyl-salicylic acid.[309,310] Further details on target levels are outlined in *Table 19*. It should be noted that failure to reach the target HbA_{1c} level should be avoided, and that early escalation of glucose-lowering therapy is essential.

Table 10 Mean efficacy of pharmacological treatment options in patients with type 2 diabetes[53,54,331]

Drug	Mean lowering of initial HbA_{1c} (%)
Alpha-glucosidase inhibitors	0.5–1.0
Biguanides	1.0–1.5
Glinides	0.5–1.5
Glitazones	1.0–1.5
Insulin	1.0–2.0
Sulphonylurea derivatives	1.0–1.5

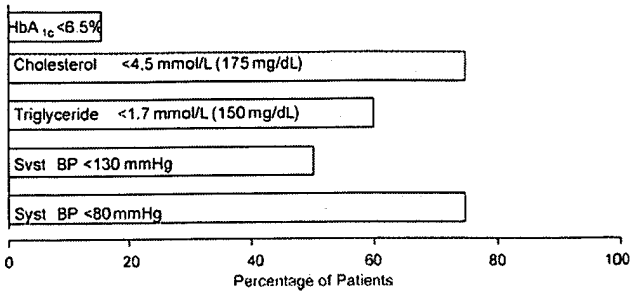


Figure 14 Percentage of patients achieving pre-defined intensive treatment targets in the Steno 2 Study (modified after Gaede *et al.*[309]).

Closing the gap between the complex needs of comprehensive management in high risk and multimorbid individuals with type 2 diabetes and the challenges in daily life, intensive counselling of patients is mandatory.[304,305] These patients are not infrequently prescribed up to 10 different classes of drugs in addition to the counselling of a healthy life style. Structured therapy, including educational classes and training programmes for acquiring the skills for a healthy life style and self-monitoring of blood glucose and blood pressure are an indispensable pre-requisite for successful management and therapy.[304,305,310-312] A mutual reviewing of the self-management protocols at each patient visit allows physicians and patients to become partners in treatment. Paramedical personnel e.g. certified diabetes educators and nurses play an integrated role in this quality process. Successful comprehensive management of patients with diabetes requires a framework of quality structures with auditing of processes and outcomes. Certified quality management should be reinforced by appropriate incentives both for the patient and the physician.

Recommendation

Structured patient education improves metabolic and blood pressure control. Class I, Level of Evidence A.

Non-pharmacological life style therapy improves metabolic control. Level A, Class I.

Self-monitoring improves glycaemic control. Class I, Level of Evidence A.

Glycaemic control

Relation to microangiopathy and neuropathy

RCTs have provided compelling evidence that diabetic microangiopathy and neuropathy can be reduced by tight glycaemic control.[71,286,287,291,309,314] This will also exert a favourable influence on CVD.[288-291,295] Nephropathy accelerates CVD and autonomic neuropathy may mask its symptoms. When compared to conventional treatment regimens, intensified treatment options, aimed at lowering haemoglobin HbA_{1c} close to the normal range, have consistently been associated with a markedly decreased frequency and extent of microvascular and neuropathic complications in people with type 1 and type 2 diabetes. Not only does this apply to primary, but also to secondary

intervention.[71,286,287,314] Analyses from the Diabetes Control and Complication Trial (DCCT) and the UKPDS demonstrated a continuous relationship between HbA_{1c} and microangiopathic complications without any apparent threshold of benefit.[287,295] In the DCCT, a 10% reduction of HbA_{1c} was associated with a 40–50% lower risk of retinopathy or its progression, although the absolute reduction in risk was substantially less at lower HbA_{1c} levels, e.g. below 7.5%. The UKPDS reported a linear relationship with each 1.0% lower HbA_{1c} associated with a 25% decline in the risk of microvascular complications, again with a rather low absolute risk at HbA_{1c} levels below 7.5%. These notions were also underlined by earlier European studies in type 1 diabetes as well as the Kumamoto and the Steno 2 studies in type 2 diabetes.[288,289,309,315] Microvascular complications, both at the kidney and the eye level, warrant meticulous further therapeutic measures, including adequate control of blood pressure with the use of ACE-inhibitors and/or angiotensin receptor-II-blockers and the cessation of smoking. Accordingly, screening for microalbuminuria and retinopathy is mandatory on an annual basis.

Relation to macroangiopathy

Although rather suggestive, the relation between macrovascular disease and hyperglycaemia is less clear than the relation to microangiopathy.[71,286,288,295,309,310,314] In fact, the recent DCCT open post-study long-term follow-up over 11 years (EDIC Study) convincingly demonstrated that a randomly assigned intervention, aiming at tight glycaemic control (mean HbA_{1c} close to 7% over the first 7–10 years), effectively reduced cardiac and other macrovascular disease manifestations from 98 events in 52 patients to 46 events in 31 patients, corresponding to a decrease of 42%.[316] The risk for MI and stroke, as well as the mortality risk from CVD, was reduced by 57%. This result was achieved despite a less tight control during the last years of follow-up when mean HbA_{1c} was around 8%. This important finding was based on a 93% follow-up rate of the original cohort of 1441 patients with type 1 diabetes and comparable levels of blood pressure and blood lipid control. The only significant confounding factor was a higher rate of microalbuminuria and macroproteinuria in the less well-controlled group (complications that are dependent on glycaemic control on their own). On statistical grounds, the reduction of HbA_{1c} was by far the most important factor behind the reduction of CVD with a 21% reduction for each percent decrease of HbA_{1c}. In type 2 diabetes, as shown by the UKPDS, each percent decline of HbA_{1c} caused a 14% lower rate of MI and fewer deaths from diabetes or any cause.[71,295]. In the Kumamoto Trial, a lower HbA_{1c} (7.0 vs. 9.0%) resulted in a cardiovascular event rate over 10 years of less than half in the control group. This difference did not, however, reach statistical significance due to small absolute numbers. Notably, the patients had a normal BMI and blood pressure.[315] A recent meta-analysis, however, in some 1800 type 1 as well as in 4472 type 2 diabetic patients, from the above-mentioned and other randomized intervention trials, convincingly showed a significant decrease of a combined incidence rate ratio for any macrovascular event down to 0.38 (95%CI 0.26–0.56) in type 1 and 0.81 (95%CI 0.73–0.91) in type 2 diabetes.[177] Finally, as mentioned earlier, the Steno 2 Study, with its multifactorial intervention, including a HbA_{1c} target below 6.5 vs. 7.5% in the control group and achieving approximately a 0.8% difference, reported a highly significant reduction of macrovascular events over 7.8 years.[309] The presence of microalbuminuria was a key inclusion criterion in the Steno 2 Study. Although all patients need appropriate therapy, screening for microalbuminuria is mandatory in all diabetic patients on an annual basis, to detect individuals at particular high-risk and to follow treatment outcome.

Nearly all prospective observational studies assessing the risk of macrovascular disease in diabetes have shown that this risk is increased already at glycaemic levels slightly above the normal range or even within the high normal range.[292,295–297] Indeed, increased cardiovascular event rates have been found in men and women with IGT.[15,62,64,296–298] In particular, plasma glucose levels 2 h after the glucose load appear to be highly predictive for cardiovascular events, even more than fasting glucose levels.[15,62,63,178] Reduction of post-prandial glucose concentrations by means of an alpha-glucosidase-inhibitor prevented the onset of overt type 2 diabetes at the stage of IGT, at least during the study period. In this trial there was also a reduction of cardiovascular events, especially MI. The number of events was, however, relatively small and although significant these results should be interpreted with great caution.[70,178] *Post hoc* analyses of randomized trials in patients with type 2 diabetes using the same alpha-glucosidase-inhibitor and with follow-up periods of at least 1 year confirmed these observations in the context of targeting meal-related hyperglycaemia.[73] It cannot be totally excluded that a reduction of insulin resistance was of importance in these studies. Insulin resistance is another strong predictor of CVD.[131,135,300] Moreover, components of the metabolic syndrome such as high blood pressure or lipid abnormalities were also attenuated by the chosen intervention in these studies targeting post-prandial hyperglycaemia.[70] Along this line, reducing both insulin resistance and HbA_{1c}, as in the PROACTIVE Trial, was associated with a 16% (absolute difference 2.1%; NNT = 49) decrease of cardiovascular endpoints such as death, MI, and stroke.[320]

Relationship with acute coronary syndromes

A wealth of reports indicate that a random blood sugar on admission for an acute coronary syndrome (ACS) is strongly correlated with the short- and long-term outcome of these patients.[293,321–324] Higher blood sugar concentrations in persons with diabetes, including those previously undiagnosed, are highly predictive for poorer outcome both in the hospital and subsequently.[70,320–324] The landmark Diabetes Glucose And Myocardial Infarction (DIGAMI) Study performed in patients with an ACS, targeted acute hyperglycaemia on admission in a randomized fashion by means of an insulin–glucose infusion. Within 24 h, glycaemia was significantly lower in the intervention group, to be maintained at a lower level during the next year. This difference translated into a 11% reduced mortality in absolute terms, indicating an NNT of nine patients for one life saved. The beneficial effect was still apparent after 3.4 years with a relative mortality reduction of about 30%.[323,325] DIGAMI 2 confirmed that glycaemic control is highly predictive for the 2-year mortality rate, but did not show any clinically relevant differences between different blood glucose-lowering regimens.[326] A recently published study, however, with a follow-up of only 3 months, confirmed that the mean

achieved blood glucose is relevant for mortality in diabetic post-MI patients, whereas insulin therapy *per se* did not lower mortality.[66]

Targeting acute hyperglycaemia in diabetic patients with ACS was also introduced into the Schwabing Myocardial Infarction Registry. Provided that all other potential interventions were equally applied to non-diabetic and diabetic patients, 24 h mortality among the diabetics was normalized and total in-hospital mortality the same for the patients with and without diabetes.[327]

Current treatment approach to glycaemic control

In type 1 diabetes, the gold standard of therapy in the post-DCCT era is intensified insulin therapy, based on appropriate nutrition and blood glucose self-monitoring, aiming at HbA_{1c} below 7%. Episodes of hypoglycaemia need to be titrated against this goal and severe hypoglycaemic episodes should be best below a rate of 15/100 patient-years.[310,328]

In type 2 diabetes, a common pharmacologic treatment approach is less well-accepted. Various diabetes associations have advocated HbA_{1c} targets below 7.0 or 6.5%, respectively (*Table 11*).[310,328,329]

Disappointingly, only a minority of patients achieved proposed glucose targets during long-term follow-up in studies like the UKPDS or Steno 2.[71,309] The greatest advance in the treatment of type 2 diabetes in recent years is the advent of polypharmacy, originally suggested by the UKPDS investigators.[330] A concept of early combination therapy has been put forward intended to maximize efficacy and minimize side-effects. It is based on the fact that a medium dose yields about 80% of the glucose-lowering effect, still minimizing potential side-effects such as weight gain, gastrointestinal discomfort, and the risk for hypoglycaemia.[331] This includes early initiation to insulin if oral glucose-lowering drugs in appropriate doses and combinations, backed by appropriate life style therapy, fails to reach target. BMI and the risks of hypoglycaemia, renal insufficiency, and heart failure are major determinants for the choice of treatment[331] (*Table 12*).

In addition, the stage of the disease and the related preponderant metabolic phenotype should be considered when tailoring therapy to individual needs (*Table 13*).[331–334]

A strategy for the selection of various glucose-lowering pharmacological options based on an assumption or, if available, more detailed knowledge on the glucometabolic situation is outlined in *Table 13*. The use of metformin has emerged as an important option both for mono- and combination therapy including insulin, provided that contraindications for this compound are absent.[291]

Table 11 Glycaemic targets for the care of patients with diabetes as recommended by various organizations[107,110,420]

Organization	HbA_{1c} (%)	FPG (mmol/L)	Post-prandial PG (mmol/L)
ADA	<7	<6.7 (120)[a]	None
IDF-Europe	≤6.5	≤6.0 (108)[a]	≤7.5 (135)[a]
AACE	≤6.5	<6.0 (108)[a]	<7.8 (140)[a]

[a]Values expressed in mg/dL.
ADA = American Diabetes Association; AACE = American College of Endocrinology; IDF = International Diabetes Federation.

Successful multicomponent glucose-lowering therapy requires self-monitoring of blood glucose to ensure that metabolic targets are met. Again, the regimen of blood glucose self-monitoring depends on the choice of therapy used and the metabolic phenotype. Obviously, when near-normoglycaemia is the goal, post-prandial glycaemia needs to be taken into account in addition to fasting glycaemia. Monnier *et al.*[313] have shown that to achieve good glycaemic control of $HbA_{1c} < 8\%$ requires measures that lower post-prandial glucose excursion, i.e. treatment that only improves the fasting glucose level will not be sufficient. Blood glucose monitoring is also advantageous in type 2 diabetic patients without insulin treatment, as evidenced by recent meta-analyses.[311,312]

There is an ever increasing body of evidence that a target close to the normal glycaemic level is advantageous for reducing CVD in people with diabetes. Still, proof of efficacy for primary prevention awaits confirmation. The glycaemic targets recommended for most persons with type 1 and type 2 diabetes are listed in *Table 11*. They should, however, be tailored to individual needs, especially in view of the risk of hypoglycaemia and other compound-specific side-effects of therapy. Immediate action, including the use of insulin infusion, is mandatory in patients with ACS and may require the joint approach of an endocrinologist and a cardiologist. In the long-term, provided it can be safely achieved, lowering blood glucose toward the normal range should be considered; this might need special targeting of post-prandial hyperglycaemia.

Table 12 Potential down-sides of pharmacological treatment modalities in patients with type 2 diabetes[331] (see also *Table 10*)

Potential problem[a]	Avoid or reconsider
Unwanted weight gain	Sulphonylureas, glinides, glitazones, insulin
Gastrointestinal symptoms	Biguanides, alpha-glucosidase inhibitors
Hypoglycaemia	Sulphonylureas, glinides, insulin
Impaired kidney function	Biguanides, sulphonylureas
Impaired liver function	Glinides, glitazones, biguanides, alpha-glucosidase inhibitors
Impaired cardio-pulmonary function	Biguanides, glitazones

[a]Oedema or lipid disorders may need further considerations.

Table 13 Suggested policy for the selection of glucose-lowering therapy according to the glucometabolic situation[331]

Post-prandial hyperglycaemia	Alpha-glucosidase inhibitors, short-acting sulphonylureas, glinides, short-acting regular insulin, or insulin analogs
Fasting hyperglycaemia	Biguanides, long-acting sulphonylureas, glitazones, long-acting insulin, or insulin analogs
Insulin resistance	Biguanides, glitazones, alpha-glucosidase inhibitors
Insulin deficiency	Sulphonylureas, glinides, insulin

Recommendation

Near normoglycaemic control reduces microvascular complications. Class I, Level of Evidence A.

Near normoglycaemia reduces macrovascular complications. Level A, Class I.

Intensified insulin therapy in type 1 diabetes improves morbidity and mortality. Class I, Level of Evidence A.

Early, stepwise increase of therapy towards pre-defined treatment targets improves a composite of morbidity and mortality in type 2 diabetes. Class IIa, Level of Evidence B.

Early initiation of insulin should be considered in patients with type 2 diabetes failing glucose target, and in patients with excessive post-prandial glucose excursions. Meal-time short-acting insulin is recommended. Class IIb, Level of Evidence C.

Metformin is recommended as first line drug in overweight type 2 diabetes. Class IIb, Level of Evidence C.

Dyslipidaemia

Table of Recommendations:

Recommendation	Class[a]	Level[b]
Elevated LDL- and low HDL-cholesterol are important risk factors in people with diabetes	I	A
Statins are first-line agents for lowering LDL-cholesterol in diabetic patients	I	A
In diabetic patients with CVD statin therapy should be initiated regardless of baseline LDL-cholesterol with a treatment target of <1.8–2.0 mmol/L (<70–77 mg/dL)	I	B
Statin therapy should be considered in adult patients with type 2 diabetes, without CVD, if total cholesterol >3.5 mmol/L (>135 mg/dL), with a treatment targeting a LDL-cholesterol reduction of 30–40%	IIb	B
Given the high lifetime risk of CVD, it is suggested that all type 1 patients over the age of 40 years should be considered for statin therapy. In patients 18–39 years (either type 1 or type 2), statin therapy should be considered when other risk factors are present, e.g. nephropathy, poor glycaemic control, retinopathy, hypertension, hypercholesterolaemia, features of the metabolic syndrome, or family history of premature vascular disease	IIb	C
In diabetic patients with hypertriglyceridaemia >2 mmol/L (177 mg/dL) remaining after having reached the LDL-cholesterol target with statins, statin therapy should be increased to reduce the secondary target of non-HDL cholesterol. In some cases, combination therapy with the addition of ezetimibe, nicotinic acid, or fibrates may be considered	IIb	B

[a]Class of recommendation.
[b]Level of evidence.

Background and epidemiology

As part of the metabolic syndrome and the pre-diabetic state, dyslipidemia in type 2 diabetes is often present at the time of diagnosis. It persists despite the use of hypoglycaemic therapy and requires specific therapy with diet, life style, and hypolipidaemic drugs. It is characterized by quantitative and qualitative changes in lipid and lipoproteins, which are correlated with insulin resistance. Typically, there is moderate hypertriglyceridaemia, low HDL cholesterol, and abnormal post-prandial lipaemia. Total and LDL cholesterol levels are similar to those in subjects without diabetes, however, LDL particles are small and dense, which is associated with increased atherogenicity and there is accumulation of cholesterol-rich remnant particles, which are also atherogenic.

Dyslipidaemia is a common finding in type 2 diabetes. The Botnia Study, for instance, included 4483 men and women aged 35–70 years, of which 1697 had diabetes and 798 IFG.[131] The prevalence of low HDL cholesterol [<0.9 mmol/L (35 mg/dL) in men and <1.0 mmol/L (39 mg/dL) in women] and/or elevated plasma triglycerides [>1.7 mmol/L (151 mg/dL)] was up to three times higher in those with diabetes and two times higher in those with IFG compared to those with normal glucose tolerance. In this and other studies the prevalence of dyslipidaemia was more pronounced in women than in men.

Dyslipidaemia and vascular risk

Although total and LDL cholesterol concentrations in patients with type 2 diabetes are similar to subjects without diabetes, they are important vascular risk factors.[335–337] Observational data from the UKPDS demonstrated that a 1 mmol/L (38.7 mg/dL) increase in LDL cholesterol was associated with a 57% increase in CVD endpoints. Low HDL cholesterol was also an important predictor of vascular disease in UKPDS, a 0.1 mmol/L (4 mg/dL) increase being associated with a 15% decrease in CVD endpoints.[336]

The independent relationship of elevated plasma triglycerides to vascular risk remains controversial. However, given the complex interactions between triglycerides and other lipoproteins and the inherent variation in triglyceride concentrations, it is clear that determining the independence of the triglyceride/vascular disease relationship by mathematical modelling, such as multivariate regression analyses is likely to be fraught with problems. In a meta-analysis of population-based cohort studies, average excess risk associated with a 1 mmol/L (89 mg/dL) increase in triglycerides was 32% in men and 76% in women.[338] When adjusted for HDL cholesterol, the excess risk was halved to 37% in women and 14% in men, but remained statistically significant. High triglyceride levels and low HDL cholesterol were significantly related to all CHD events and to coronary mortality in a large cohort of patients with type 2 diabetes followed for 7 years.[339] Factor analysis and principal component analysis have shown that a 'hyperinsulinaemia cluster' (a factor having high positive loadings for BMI, triglycerides, and insulin and a high negative loading for HDL cholesterol) was predictive of CHD mortality in type 2 diabetes.[340]

Treatment benefits of statin therapy

The introduction of the statin drugs, which proved to be well-tolerated and highly effective, enabled definitive RCTs of LDL cholesterol-lowering to be undertaken. These

drugs are specific, competitive inhibitors of the enzyme 3-hydroxy 3-methylglutaryl coenzyme A reductase (HMG CoA reductase), which catalyzes the major rate determining step in cholesterol synthesis. As a result, hepatic LDL receptor activity increases with consequent increased uptake of LDL cholesterol and decreased plasma LDL-cholesterol. The statins are the most potent drugs for lowering plasma LDL-cholesterol.

Secondary prevention

Although no major secondary prevention trial has been performed in a specific diabetic population, there is sufficient evidence from *post hoc* subgroup analysis of over 5000 patients with diabetes, included in the major trials, to conclude that they show similar benefits in reduction of events (both coronary events and stroke) as patients free from diabetes.

The first landmark secondary trial was the Scandinavian Simvastatin Survival Study (4S), which included patients ($n = 4444$) with established CAD and total cholesterol concentrations between 5.5 and 8 mmol/L (193 and 309 mg/dL) despite dietary therapy.[341] The study was powered on overall mortality. This study was a randomized, placebo-controlled trial comparing simvastatin to placebo. The goal of therapy in the simvastatin group was total cholesterol between 3.0 and 5.2 mmol/L (116 and 201 mg/dL). Thirty seven percent of patients required simvastatin 40 mg/day to achieve target cholesterol levels. Simvastatin therapy was associated with a 35% reduction in LDL-cholesterol. After a median follow-up of 5.4 years, simvastatin therapy resulted in a risk reduction in overall mortality of 30% (HR 0.07; 95%CI 0.59–0.85; $P = 0.0003$). Two *post hoc* analyses of 4S involving patients with diabetes have been reported. At baseline, 202 patients (mean age 60 years, 78% male) were known to have diabetes, a small number and perhaps an atypical diabetic population given that they were hypercholesterolaemic and the triglyceride entry criteria was relatively low at <2.5 mmol/L (220 mg/dL). Lipid changes in this diabetic subgroup were similar to those observed overall. Diabetic patients on placebo had a high risk of subsequent events, approximately one-half having a major cardiovascular event during the study period. Simvastatin therapy was associated with a 55% reduction in major coronary events ($P = 0.002$). The number of diabetic patients was insufficient to examine the impact of simvastatin on the primary endpoint of overall mortality, although there was a non-significant 43% reduction.[342] A further analysis of 4S identified 483 diabetic patients by baseline plasma glucose. In this cohort, there was a significant 42% reduction in major coronary events and a 48% reduction in revascularizations.[343]

These initial results have been supported by subsequent secondary prevention trials, particularly the Heart Protection Study (HPS; *Table 14*). It is clear that patients with diabetes show similar RRRs compared to those without diabetes. Given the higher absolute risk in these patients, the NNT to prevent a CVD event is lower. However, the residual risk in diabetic patients remains high, despite statin treatment. For example, in HPS, the residual risk during the study period was higher in diabetic patients with vascular disease treated with simvastatin compared to patients with vascular disease, but no diabetes treated with placebo.[344]

When the results of the statin trials are related to the degree of LDL reduction, the results show a roughly linear relationship. More recently, the potential added benefit of achieving LDL cholesterol concentrations lower than levels previously achieved has been tested. In the Pravastatin or Atorvastatin Evaluation and Infection Therapy (PROVE-IT) Trial, standard statin therapy (pravastatin 40 mg/day) was compared to intensive therapy (atorvastatin 80 mg/day) in 4162 patients within 10 days of an ACS, with a mean

Table 14 Subgroups of patients with DM in the major secondary prevention trials with statins and the proportionate risk reduction in patients with and without diabetes[112,123,153,154,341,342,344]

Variable			Proportion of events (%)		Relative risk reduction (%)	
Trial	Type of event	Treatment	Diabetes		Patient group	
			No	Yes	All	Diabetes
4S Diabetes ($n = 202$)	CHD death or non-fatal MI	Simvastatin	19	23	32	55
		Placebo	27	45		
4S Reanalysis Diabetes ($n = 483$)	CHD death or non-fatal MI	Simvastatin	19	24	32	42
		Placebo	26	38		
HPS Diabetes ($n = 3050$)	Major coronary event, stroke, or revascularization	Simvastatin	20	31	24	18
		Placebo	25	36		
CARE Diabetes ($n = 586$)	CHD death or non-fatal MI	Pravastatin	12	19	23	25
		Placebo	15	23		
LIPID Diabetes ($n = 782$)	CHD death, non-fatal MI, revascularization	Pravastatin	19	29	24	19
		Placebo	25	37		
LIPS Diabetes ($n = 202$)	CHD death, non-fatal MI, revascularization	Fluvastatin	21	22	22	47
		Placebo	25	38		
GREACE Diabetes ($n = 313$)	CHD death, non-fatal MI, UAP, CHF revascularization, stroke	Atorvastatin	12	13	51	58
		Standard care	25	30	–	–

4S, Scandinavian Simvastatin Survival Study; HPS, Heart Protection Study; CARE, Cholesterol and Recurrent Events Trial; LIPID, Long-Term Intervention with Pravastatin in Ischaemic Disease Study; LIPS, Lescol Intervention Prevention Study; GREACE, Greek Atorvastatin and CHD Evaluation Study.
CHD = coronary heart disease; CHF = congestive heart failure; MI = myocardial infarction; revasc = revascularisation; UAP = unstable angina pectoris.

follow-up of 24 months.[345] More intensive therapy [achieved mean LDL 1.6 mmol/L (62 mg/dL)] was associated with a significant 16% risk reduction in cardiovascular events, compared to standard therapy [mean LDL 2.5 mmol/L (97 mg/dL)]. PROVE-IT included 734 diabetic patients (18%) and there was no heterogeneity of effect in this subgroup.

Treat to New Targets Trial (TNT) has reported on the effects of intensive statin therapy (atorvastatin 80 mg/day), compared to standard therapy (atorvastatin 10 mg/day), in 10 001 patients with stable CAD.[346] Intensive therapy [mean LDL cholesterol 2.0 mmol/L (77 mg/dL)] was associated with a 22% risk reduction compared to standard therapy [mean LDL cholesterol 2.6 mmol/L (101 mg/dL)], over a median follow-up of 4.9 years. In a recent subgroup analysis of the TNT, the results of intensive, compared to standard, atorvastatin therapy were reported for the 1501 patients with diabetes, 735 received atorvarstatin 10 mg/day, and 748 atorvastatin 80 mg/day. By the end-of-treatment, mean LDL cholesterol levels were 2.6 mmol/L (99 mg/dL) with atorvastatin 10 mg and 2.0 mmol/L (77 mg/dL) with atorvastatin 80 mg. A primary event occurred in 135 patients (17.9%) receiving atorvastatin 10 mg, compared with 103 patients (13.8%) receiving atorvastatin 80 mg (HR 0.75, $P = 0.026$). Significant differences between the groups in favour of atorvastatin 80 mg were also observed for time to cerebrovascular event [0.69 (0.48–0.98), $P = 0.037$] and any cardiovascular event [0.85 (0.73–1.00), $P = 0.044$].[181]

Goals of therapy for secondary prevention

Based on evidence from RCTs, the Third Joint European Societies Task Force on Cardiovascular Disease Prevention in Clinical Practice[347] recommended treatment goals for patients with established CVD of total cholesterol <4.5 mmol/L (174 mg/dL) and LDL-cholesterol <2.5 mmol/L (97 mg/dL). This LDL goal is similar to that of the Adult Treatment Panel III (ATP III) of the Cholesterol Education Programme in the USA.[348] More recently, guidelines have been reviewed by the National Cholesterol Education Programme, in the light of recent RCTs.[348] Thus, for very high-risk patients, including those with diabetes and symptomatic CVD, a therapeutic option of an LDL goal ≤1.8 mmol/L (70 mg/dL) is suggested.

Recommendation

Elevated LDL- and low HDL-cholesterol are important risk factors in people with diabetes. Class I, Level of Evidence A.

Statins are first-line agents for lowering LDL-cholesterol in diabetic patients. Class I, Level of Evidence A.

In diabetic patients with CVD, statin therapy should be initiated regardless of baseline LDL-cholesterol with a treatment target of ≤1.8 mmol/L. Class I, Level of Evidence B.

Primary prevention

Given the high risk of CVD in diabetic patients, together with a higher mortality associated with the first event, primary prevention with lipid-lowering is an important component of global preventive strategies in patients with type 2 diabetes.

Information from RCTs is available to inform clinical decisions from large cohorts of diabetic patients included in HPS[344] and the Anglo-Scandinavian Cardiac Outcomes Trial-Lipid Lowering Arm (ASCOT-LLA).[349] In ASCOT-LLA, 10 mg of atorvastatin was compared with placebo in 10 305 hypertensive patients with non-fasting total cholesterol of 6.5 mmol/L (251 mg/dL) or less, of whom 2532 had type 2 diabetes. Atorvastatin therapy was associated with a 36% reduction in the primary endpoint of non-fatal MI and fatal CAD, after a median follow-up of 3.3 years. Tests for heterogeneity showed that those with diabetes ($n = 2532$) responded in a similar way, although there were too few events ($n = 84$) to assess reliably the effect in the subgroup alone. In HPS, there were 2912 diabetic patients without symptomatic vascular disease.[344] In this cohort, the risk reduction was 33% ($P = 0.0003$) with simvastatin 40 mg/day.

The Collaborative Atorvastatin Diabetes Study (CARDS), compared atorvastatin 10 mg to placebo, in a population of patients with type 2 diabetes (aged 40–75 years) without high cholesterol [baseline LDL 3.0 mmol/L (116 mg/dL)], but with one other risk factors for CVD: hypertension, retinopathy, proteinuria, or cigarette smoking. After a median follow-up of 3.9 years, the risk reduction in first major cardiovascular events was 37% ($P = 0.001$). In all three trials there was no heterogeneity of effect with regard to baseline LDL-cholesterol or other lipid values.[350]

Goals of therapy for primary prevention

In the Joint European Guidelines, similar goals of therapy are given for diabetic patients for primary prevention, as for patients with symptomatic disease: cholesterol <4.5 mmol/L (<174 mg/dL) and LDL <2.5 mmol/L (<97 mg/dL). Patients with type 1 diabetes and proteinuria are included in this guidance.[347] In ATP III, most patients with diabetes without symptomatic disease are considered at high risk and an LDL goal of <2.6 mmol/L (100 mg/dL) is suggested. Given that, diabetic patients in HPS and CARDS with low LDL cholesterol levels showed similar relative benefit with statin therapy to those with higher LDL levels, an important clinical question is whether to start statin therapy in patients whose LDL cholesterol is already <2.6 mmol/L (<100 mg/dL). Currently, this decision is left to clinical judgment.[346] In those diabetic patients considered to be at lower risk, drug therapy might not be started if LDL-cholesterol is <3.4 mmol/L (<131 mg/dL). The most recent guidance from the ADA suggests that, in patients with diabetes and a total cholesterol >3.5 mmol/L (>135 mg/dL), statin therapy to achieve an LDL reduction of 30–40% regardless of baseline LDL levels is recommended.[351]

In patients with type 1 diabetes, who also have a high lifetime risk of CVD, evidence is still lacking regarding the role of statin therapy for primary prevention.

Recommendation

Statin therapy should be considered in adult patients with type 2 diabetes, without CVD, if total cholesterol >3.5 mmol/L (>135 mg/dL), with treatment aiming for an LDL-cholesterol reduction of 30–40%. Class IIb, Level of Evidence B.

Given the high lifetime risk of CVD, it is suggested that all type 1 patients over the age of 40 years should be considered for statin therapy. In patients 18–39 years (either type 1 or type 2), statin therapy should be considered when other risk factors are present, e.g. nephropathy, poor glycaemic control, retinopathy, hypertension, hypercholesterolaemia, features of the metabolic syndrome or

family history of premature vascular disease. Class IIb, Level of Evidence C.

Fibrate trials

There is much less information available from RCTs to determine clinical practice in terms of fibrate therapy compared to statin therapy. A small number of diabetic patients ($n = 135$) were included in the Helsinki Heart Study (HHS), a primary prevention trial in 4082 men with non-HDL cholesterol (total cholesterol minus HDL cholesterol) >5.2 mmol/L (201 mg/dL) comparing gemfibrozil to placebo. Overall, gemfibrozil was associated with a significant 35% risk reduction. In a *post hoc* analysis of individuals with a cholesterol/HDL ratio >5 and triglycerides >2.3 mmol/L (>204 mg/dL), there was a 71% reduction in risk. The incidence of non-fatal MI and coronary death was significantly higher during the trial in the small diabetic cohort, but the 68% risk reduction observed with gemfibrozil did not reach statistical significance, given the small numbers.[352]

In the Veterans Administration HDL Trial (VAHIT), gemfibrozil was compared with placebo in 2531 men with stabilized CAD and low HDL cholesterol [baseline HDL 0.8 mmol/L (31 mg/dL)] and a relatively normal LDL [baseline LDL 2.8 mmol/L (108 mg/dL)]. After a mean follow-up of 5.1 years, gemfibrozil therapy was associated with a 22% risk reduction in the primary endpoint of non-fatal MI or coronary death ($P = 0.006$). In a subgroup of 309 diabetic patients, a composite endpoint of coronary death, stroke, and MI, was reduced by 32% (coronary death by 41% and stroke by 40%). This trial suggests benefit beyond LDL-lowering, in that gemfibrozil therapy did not change LDL cholesterol, but HDL cholesterol increased by 6% and triglycerides fell by 31%.[353,354]

The FIELD Study (Fenofibrate Intervention and Event Lowering in Diabetes) assessed the effect of fenofibrate (micronized preparation 200 mg/day) compared to placebo in type 2 diabetes, with ($n = 2132$) and without ($n = 7664$) previous CVD.[355] After a mean duration of 5 years, fenofibrate therapy was associated with an RRR of 11% (HR 0.89, 95%CI 0.75–1.05) in the primary endpoint of CHD death and non-fatal MI, which did not reach statistical significance ($P = 0.16$). Non-fatal MI was reduced significantly (HR 0.76, 95%CI 0.62–0.94; $P = 0.01$), but CHD mortality showed a non-significant increase (HR 1.19, 95%CI 0.90–1.57; $P = 0.22$). Total cardiovascular events (cardiac death, MI, stroke, coronary and carotid revascularization) were significantly reduced by fenofibrate therapy ($P = 0.035$). Total mortality was 6.6% in the placebo and 7.3% in the fenofibrate group ($P = 0.18$). In a *post hoc* analysis, fenofibrate therapy was associated with a reduction in coronary events in patients with no previous CVD, but not in those with previous CVD ($P = 0.03$ for interaction).

There has been much conjecture concerning the conflicting results of FIELD. The degree of baseline dyslipidaemia [total cholesterol 5.0 mmol/L (195 mg/dL), total triglyceride 2.0 mmol/L (173 mg/dL), LDL cholesterol 3.1 mmol/L (119 mg/dL), and HDL cholesterol 1.1 mmol/L (43 mg/dL)], was possibly insufficient to demonstrate the optimal effect of the drug. In the Veterans Administration HDL Trial, a secondary prevention trial which demonstrated a positive outcome with gemfibrozil, baseline HDL cholesterol was 0.8 mmol/L. Other possible confounders include the higher drop-in therapy with statins in the placebo group, the potentially adverse effect of fenofibrate on homocysteine levels (an increase of 3.7 μmol/L) and the relatively small impact in reducing LDL cholesterol and increasing HDL cholesterol (only 2% by the end of the study). However, the major conclusion following the results of FIELD trial is that guidance on treatment strategies remains unchanged and statins remain the major treatment choice in the majority of diabetic patients.

Guidelines for HDL cholesterol and triglycerides

Given the paucity of information available from controlled trials, guidelines are less specific with regard to goals for HDL cholesterol and triglycerides. However, the joint European guidelines recognize low HDL cholesterol [<1 mmol/L (39 mg/dL) in men and <1.2 mmol/L (46 mg/dL) in women] and fasting triglycerides >1.7 mmol/L (151 mg/dL) as markers of increased vascular risk. In the recent update of ATP III for patients considered at very high risk, such as diabetic patients with symptomatic vascular disease, and high triglyceride and low HDL cholesterol consideration can be given to combining a fibrate or nicotinic acid with an LDL-lowering drug.[348] When triglycerides are >2.3 mmol/L (>189 mg/dL) but LDL cholesterol levels are to goal following statin therapy, a secondary treatment target of non-HDL cholesterol (total cholesterol minus HDL cholesterol) is suggested with a goal 0.8 mmol/L (31 mg/dL) higher than the identified LDL cholesterol goal.

Recommendation

In diabetic patients with hypertriglyceridaemia >2 mmol/L (177 mg/dL) remaining after having reached the LDL-cholesterol target, it is recommended that statin therapy should be increased to reduce non-HDL cholesterol with a goal of therapy 0.8 mmol/L (31 mg/dL) higher than that identified for LDL. In patients on maximum dose, or maximum tolerated dose of statin, where LDL-C or non-HDL-C is not to goal, the addition of ezetimibe, a specific inhibitor of cholesterol absorption, should provide effective further cholesterol reduction. In some cases combination therapy with nicotinic acid, or fibrates may be considered. Class IIb, Level of Evidence B.

Future views

Diabetic dyslipidaemia is an important CVD risk factor that is open to therapeutic intervention. Much information is now available from RCTs to guide clinical practice, particularly with statins. On-going trials will provide further information concerning the added benefit of lowering LDL-cholesterol to levels below current guidelines. However, it seems reasonable, given the very high risk of diabetic patients with established CVD, that the new lower target of <1.8 mmol/L (<70 mg/dL) LDL suggested by ATP III should be adopted. For primary prevention, the new guidelines from the ADA, produced after the publication of HPS and CARDS, suggesting statin therapy regardless of baseline LDL to achieve a 30–40% reduction, is reasonable.

The on-going ACCORD Trial, will provide evidence for statin/fibrate combination in diabetes. Another statin combination, for instance with nicotinic acid, which has proved successful in atheroma regression trials, is likely to be considered given the HDL-increasing properties of nicotinic acid at doses less likely to impair insulin resistance. In the longer term, specific HDL-increasing drugs will

enable the HDL hypothesis to be tested in formal randomized clinical trials.

Blood pressure

Table of Recommendations:

Recommendation	Class[a]	Level[b]
In patients with diabetes and hypertension, the recommended target for blood pressure control is <130/80 mm Hg	I	B
The cardiovascular risk in patients with diabetes and hypertension is substantially enhanced. The risk can be effectively reduced by blood pressure-lowering treatment	I	A
The diabetic patient usually requires a combination of several anti-hypertensive drugs for satisfactory blood pressure control	I	A
The diabetic patient should be prescribed a RAS inhibitor as part of the blood pressure lowering treatment	I	A
Screening for microalbuminuria and adequate blood pressure-lowering therapy including the use of ACE-inhibitors and angiotensin receptor-II-blockers improve micro- and macrovascular morbidity in type 1 and type 2 diabetes	I	A

[a]Class of recommendation.
[b]Level of evidence.

Background

Hypertension is up to three times more common in patients with type 2 DM than in non-diabetic subjects,[356,357] and is frequent in patients with type 1 diabetes as well. In the latter condition, nephropathy usually precedes hypertension, which then accelerates the progress of micro- and macrovascular complications. Obesity, increasing age, and onset of renal disease further increase the prevalence of hypertension in diabetic patients.[358]

Diabetes and hypertension are additive risk factors for atherosclerosis and CVD, and hypertension enhances the risk for such disease, more in patients with diabetes than in hypertensive normoglycaemic subjects, as demonstrated for instance by the Multiple Risk Factor Intervention Trial[359,360] and the PROspective CArdiovascular Munster (PROCAM) Study.[361] There are several possible reasons for this increased risk, one being enhanced susceptibility to pressure-induced vascular wall stress. The diabetic myocardium may also be more sensitive to other risk factors for CVD, increasing the risk for myocardial hypertrophy, ischaemia, and heart failure.[362] Furthermore, diabetic nephropathy is incrementally accelerated by a raised blood pressure creating a vicious cycle once hypertension and nephropathy are present.[363] It should be noted that renal artery stenosis may be responsible for both renal insufficiency and hypertension in the diabetic patient. Screening for this condition is warranted in patients with refractory hypertension and/or renal insufficiency.

Treatment targets

Measures to lower raised blood pressure should be particularly aggressive in patients with either type 1 and type 2 diabetes, because of the substantially enhanced cardiovascular risk associated with increasing blood pressure levels. The UKPDS and the Hypertension Optimal Treatment (HOT) Study revealed that an intensive blood pressure-lowering treatment strategy is associated with a lower incidence of cardiovascular complications in patients with diabetes.[364,365] Various manifestations of CVD, including stroke and renal disease, were markedly reduced in diabetic patients randomized to rigorous blood pressure control in comparison with those randomized to a less tight control. There is a general consensus that recommended blood pressure targets should be lower in patients with (<130/80 mm Hg) than in those without diabetes (<140/90 mm Hg). If tolerated, diabetic patients with nephropathy should be treated towards even lower blood pressure levels. A vigorous lowering of blood pressure may initially elevate serum creatinine, but will benefit renal function in a long-term perspective.

Recommendation

In patients with diabetes and hypertension, the recommended target for blood pressure control is <130/80 mm Hg. Class I, Level of Evidence B.

How should blood pressure be lowered?

As already outlined in these guidelines, life style interventions treatment should form the basis in the treatment of all patients with hypertension (see section on life style and comprehensive management). Although important, life style-oriented changes are usually insufficient for adequate blood pressure control. Most patients need some form of pharmacological treatment, and a combination of several blood pressure-lowering drugs is frequently needed to achieve satisfactory blood pressure control. Registries and clinical trials reveal that many patients with diabetes still do not reach the recommended target of a blood pressure <130 mm Hg systolic and <80 mm Hg diastolic.[366,367] Thus, there is a considerable potential for improved patient management in this respect. Only a few large prospective, randomized clinical trials with anti-hypertensive agents have specifically been oriented towards patients with diabetes. However, several large placebo-controlled trials with sizeable subgroups of patients with diabetes have reported specifically on the outcome in that subgroup (*Table 15*). A consistent finding in these analyses is a marked reduction of the risk for subsequent cardiovascular events among patients on active treatment compared to those on placebo. This finding is consistent for all types of blood pressure-lowering drugs that have been studied.

Chosen as the initial drug, the beneficial effect of diuretics, beta-blockers (BB), calcium channel blockers (CCB), and ACE-inhibitors are well documented.[368-373] More recently, different anti-hypertensive drugs have been compared to each other (*Table 16*). In some of these studies, it appears that blockade of the renin-angiotensin-aldosterone system seems to be of particular value, especially when treating hypertension in patients with diabetes at particularly high cardiovascular risk.[374-376] Recent evidence supports the efficacy of an ACE-inhibitor rather than a CCB as initial therapy, when the intention is to prevent or retard the occurrence of microalbuminuria in hypertensive patients with diabetes.[377] In the Losartan Intervention For Endpoint reduction in hypertension

Table 15 Treatment effects of anti-hypertensive drugs in comparison with placebo or less-intensive treatment as reported in randomized clinical trials

Trial (Reference no.)	Treatment comparison	Primary outcome variable	Relative risk reduction		Absolute risk reduction	
			Diabetes (%)	No diabetes (%)	Diabetes (%)	No diabetes (%)
HDFP[383]	Diuretic vs. standard therapy	All-cause mortality	27	21	4.2	3.0
SHEP[368]	Diuretic vs. placebo	Stroke	54	23	8.8	3.1
Syst-EUR[370]	CCB vs. placebo	Stroke	69	36	18.3	4.5
HOT[365]	<80 mm Hg DBP vs. <90 mm Hg DBP	MI/stroke/CV-mortality	51	11	12.5	1.0
HOPE[372,373]	ACE-I vs. placebo	MI/stroke/CV-mortality	25	21	4.5	2.2

ACE-I = angiotensin-converting-enzyme inhibitor; BB = beta-blocker; CCB = calcium channel blocker; DBP = diastolic blood pressure; CV = cardiovascular.

Table 16 Treatment effects expressed in HR (95%CI) in randomized clinical trials comparing different anti-hypertensive treatments in hypertensive patients with type 2 diabetes

Trial (Reference no.)	Treatment comparison	*n*	CAD	Effect on various outcome variables		CV mortality
				Stroke	Mortality	
UKPDS[364]	ACE-I vs. BB	1148	ns	ns	ns	ns
FACET[375]	ACE-I vs. CCB	380	ns	ns	ns	ns
ABCD[374]	ACE-I vs. CCB	470	0.18 (0.07–0.48)	ns	ns	ns
CAPPP[376]	ACE-I vs. BB/Tz	572	0.34 (0.17–0.67)	ns	0.54 (0.31–0.96)	0.48 (0.21–1.10)
STOP-2[384]	ACE-I vs. BB/Tz	488	0.51 (0.28–0.92)	ns	ns	ns
STOP-2[384]	CCB vs. BB/Tz	484	ns	ns	ns	ns
NORDIL[385]	CCB vs. BB/Tz	727	ns	ns	ns	ns
INSIGHT[386]	CCB vs. BB/Tz	1302	ns	ns	ns	ns
ALLHAT[380]	ACE-I vs. Tz	6929	ns	ns	ns	nr
ALLHAT[380]	CCB vs. Tz	7162	ns	ns	ns	nr
LIFE[378]	ARB/Tz vs. BB/Tz	1195	ns	0.79 (0.55–1.14)	0.61 (0.45–0.84)	0.63 (0.42–0.95)
ASCOT[387]	CCB/ACE-I vs. BB/Tz	5145	nr	Combined major cardio-vascular events 0.86 (0.76–0.98)		

ARB = angiotensin receptor blocker; CAD = coronary artery disease (mainly myocardial infarction); CV = cardiovascular; ACE-I = angiotensin-converting-enzyme inhibitor; BB = beta-blocker; CCB = calcium channel blocker; Tz = thiazide (or thiazide-like) diuretic; ns = not significant; nr = not reported.

Study (LIFE), recruiting patients at high risk due to established LV hypertrophy, blood pressure-lowering therapy initiated with the angiotensin receptor blocker (ARB), losartan, was more effective in reducing the primary composite cardiovascular endpoint than the selective BB atenolol. In this study, the beneficial effect of losartan was even more apparent in the diabetic subpopulation, with a statistically significant difference also in all-cause mortality.[378] It should be noted that the vast majority of patients in both groups received hydrochlorothiazide in addition to the ARB or BB.

As outlined in *Table 15*, the absolute risk reduction (ARR) caused by treatment of hypertension in patients with diabetes is consistently greater than in those without. The main aim when treating hypertension in diabetic patients is, therefore, to reduce blood pressure, whereas it seems less important by means of which drug or combination of drugs this is accomplished. An inhibitor of the renin-angiotensin-aldosterone system should, however, be part of the pharmacological combination. It is important to monitor renal function when instituting an ACE-inhibitor or an ARB, especially considering the risk of deterioration of renal function in the presence of renal artery stenosis.[182]

A matter that has been intensively discussed over the last decades is whether the metabolic actions of various blood pressure-lowering drugs are important for long-term cardiovascular outcome. It is well established that the use of thiazides and BBs are associated with an increased risk of developing type 2 diabetes as compared to treatment with CCBs and inhibitors of the renin-angiotensin-aldosterone system.[379,380] It is, however, not known whether treatment with BBs and/or thiazides in patients with established type 2 diabetes has any metabolic adverse events of clinical importance, including an increased risk for cardiovascular events. In the Anti-hypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial (ALLHAT), the outcome was similar in subgroups treated with a diuretic, an ACE-inhibitor, or a CCB.[381] However, in that study, the subgroup of patients with IFG was very small in comparison with the diabetic and normoglycaemic subgroups. Thus, while drugs with negative metabolic effects, especially the combination of a thiazide and a BB, probably should be avoided as first line treatment when managing hypertensive patients with the metabolic syndrome, the goal of lowering blood pressure seems more important than minor alterations in the metabolic condition in patients with established diabetes.[382] A recent observation, of potential interest in explaining differences between atenolol/thiazide-based, compared with amlodipine/perindopril-based, blood pressure-lowering therapy was

recently suggested in a substudy to ASCOT.[155] The BB/thiazide-based treatment did not lower central blood pressure to the same extent as the other combination of drugs. It was proposed, that this may relate to a diminished cardiovascular protection of the former drug combination.

Recommendation

The cardiovascular risk in patients with diabetes and hypertension is substantially enhanced. The risk can be effectively reduced by blood pressure-lowering treatment. Class I, Level of Evidence A.

The diabetic patient usually requires a combination of several anti-hypertensive drugs for satisfactory blood pressure control. Class I, Level of Evidence A.

The diabetic patient should be prescribed a RAS inhibitor as part of the blood pressure-lowering treatment. Class I, Level of Evidence A.

Screening for microalbuminuria and adequate blood pressure-lowering therapy including the use of ACE-inhibitors and angiotensin receptor-II-blockers, improves micro- and macrovascular morbidity in type 1 and type 2 diabetes. Class I, Level of Evidence A.

Management of CVD

Coronary artery disease

Table of Recommendations:

Recommendation	Class[a]	Level[b]
Early risk stratification should be part of the evaluation of the diabetic patient after ACS	IIa	C
Treatment targets, as listed in *Table 19*, should be outlined and applied in each diabetic patient following an ACS	IIa	C
Patients with AMI and diabetes should be considered for thrombolytic therapy on the same grounds as their non-diabetic counterparts	IIa	A
Whenever possible, patients with diabetes and ACS should be offered early angiography and mechanical revascularization	IIa	B
BBs reduce morbidity and mortality in patients with diabetes and ACS	IIa	B
Aspirin should be given for the same indications and in similar dosages to diabetic and non-diabetic patients	IIa	B
Adenosine diphosphate (ADP) receptor-dependent platelet aggregation inhibitor (clopidogrel) may be considered in diabetic patients with ACS in addition to aspirin	IIa	C
The addition of an ACE-inhibitor to other therapies reduces the risk for cardiovascular events in patients with diabetes and established CVD	I	A
Diabetic patients with AMI benefit from a tight glucometabolic control. This may be accomplished by different treatment strategies	IIa	B

[a]Class of recommendation.
[b]Level of evidence.

Epidemiology

Diabetes and ACS

Diabetes is common among patients with ACS, whether diagnosed as AMI or unstable angina pectoris (UAP). In the Swedish Registry for Coronary Care, about 21% of patients with AMI are recorded as having known diabetes.[388] The corresponding proportion ranges from 19 to 23% in the recent GRACE, OASIS, and EHS-ACS multinational registries.[389-391] When patients with AMI, but without known diabetes, were challenged with an OGTT, 65% had an abnormal glucose regulation (previously undiagnosed diabetes = 25% and IGT = 40%), a much higher proportion than among age- and gender-matched healthy controls, among whom 65% had a normal glucose regulation (NGR).[392,393] The prevalence of previously known and newly recognized diabetes based on fasting hyperglycaemia reached 28% in the EUROASPIRE II population.[394] The Euro Heart Survey on Diabetes and the Heart, recruiting patients from 25 countries, disclosed unrecognized diabetes in 22% of patients acutely admitted for CAD when applying an OGTT, according to WHO.[4,395] Based on these data, the overall proportion of DM among patients with ACS was estimated to be about 45%.[396]

As has been described earlier (see section on Identification of subjects at high risk for CVD and diabetes), the prevalence of DM is increasing rapidly in the general population. Accordingly, it may be foreseen that diabetes will have a substantial impact on future morbidity and mortality in patients with ACS and also on health care expenditure for such disease.

Prognostic implications

In-hospital and long-term mortality after MI has declined over the years. Unfortunately, patients with diabetes have not benefited from improvements in health care to the same extent as those without this disease. In fact, the relative impact of diabetes on cardiovascular mortality is unchanged or even increasing.[106] Patients with previously known diabetes admitted with ACS, have higher in-hospital mortality (11.7, 6.3, and 3.9% in MI with and without ST-elevation and UAP, respectively), than patients without diabetes (6.4, 5.1, and 2.9%) included in the GRACE registry.[389] This unfavourable prognosis has persisted over time and is also reflected in long term mortality, as outlined in *Table 17* as reported by studies performed at different periods of time and inspite of the introduction of improved treatment options.[388,391,397-406]

Diabetes is associated with high total mortality, accounting for 7-18% at 30 days, 15-34% after 1 year, and up to 43% after 5 years.[388,391,397-406] The relative risk for overall mortality, following adjustment for differences in baseline characteristics, concomitant diseases and baseline treatment, that is attributable to diabetes, varies between different studies, ranging from 1.3 to 5.4.[391,397-406] This risk is somewhat higher among women than men.[388,391,399] Patients with newly detected type 2 diabetes have similar proportions of re-infarction, stroke, and 1-year mortality following an AMI as patients with previously established diabetes.[406]

The main complications in patients with ACS include recurrent myocardial ischaemia, LV dysfunction, symptomatic heart failure, electrical instability (ventricular fibrillation, ventricular tachycardia, atrio-ventricular block, and sudden cardiac death), cardiogenic shock, re-infarction,

stroke, or death. Most of these complications are significantly more common in patients with diabetes. Based on a cohort of more than 11 000 survivors of AMI, the GISSI prevenzione investigators identified 12 independent predictors of long-term mortality [relative risk (CI)]. The risk factors with the strongest negative influence on prognosis were age [65–69 years: males 2.1 (1.7–2.7), females 3.3 (1.4–7.9)]; LV dysfunction [males 2.0 (1.8–2.3), females 2.0 (1.5–2.8)]; intermittent claudication [males 1.6 (1.3–2.0), females 3.3 (1.9–5.8)]; continued smoking [males 1.5 (1.2–1.7), females 2.5 (1.4–4.4)], and diabetes [males 1.3 (1.1–1.5), females 1.9 (1.4–2.7)].[407] Generalized atherosclerosis is common in type 2 diabetes; clinically manifested peripheral artery disease is three to four times more common and the incidence of cerebrovascular disease about doubled, as can be noted reviewing the baseline characteristics of patients included in various studies.[388,394,401,403,405,406]

The markedly increased adjusted risk of death associated with diabetes beyond the acute phase of coronary events indicates the profound role of the gluco-metabolic derangement. Dysglycaemia at any level[406] causes alterations in energy substrate metabolism, including insulin resistance, increased concentrations of non-esterified fatty acids, and excessive oxidative stress.[201,408] These metabolic factors are further enhanced at the onset of an AMI, when chest pain, breathlessness, and anxiety cause a stress-induced increase in adrenergic tone. As outlined more in detail in the pathophysiology chapter, diabetic patients often have a widespread and diffuse CAD, decreased vasodilatory reserve, decreased fibrinolytic activity, elevated platelet aggregability, autonomic dysfunction, and possibly diabetic cardiomyopathy, all factors to be taken into account when choosing therapy. Impaired glucose control may operate in the long-term as well. In type 2 diabetes, metabolic control measured as fasting blood glucose or glycated haemoglobin (HbA_{1c}) is a major risk factor for future CHD. Furthermore, a high blood glucose level at admission is a powerful predictor for in-hospital and long-term mortality, both in patients with and without DM.[327,409–411]

Heart failure is a common complication in survivors of an acute coronary event occurring with almost a linear time-dependent proportion (1.3% yearly) with almost 5% in the first week and 6.3% after 6 months.[412,413] The incidence of heart failure is a strong predictor of subsequent mortality, markedly increasing the risk up to 10-fold, as reported in the most recent publications from the OASIS-2 and Cholesterol and Recurrent Events Substudy (CARE) studies.[391,400,404,412–414]

Treatment principles

Revascularization

Studies on accurately characterized diabetic patients with CVD, including precise information on how the condition was diagnosed and treated, are limited. Prospective randomized, large clinical trials usually recruited low numbers of patients with diabetes due to exclusion and inclusion criteria and studies on pure diabetic populations are sparse. Thus, most information on the treatment of diabetic patients with ACS is derived from retrospective subgroup analyses and patient registries. Several registry studies show that diabetic patients are not as well-treated as non-diabetic patients, with regard to evidence-based therapy and coronary interventions.[324,404] In particular, it seems that heparins, thrombolytic agents, and coronary interventions are less frequently administered. One explanation may, as a consequence of autonomic neuropathy, be lack of typical symptoms in diabetic patients with coronary ischaemia. The reported prevalence of silent ischaemia is 10–20% in diabetic, compared with 1–4% in non-diabetic populations.[263] Accordingly, silent infarctions or infarctions with atypical symptoms are more common in diabetic patients, prolonging time to hospital admission as well as to diagnosis, thereby reducing the opportunity to administer adequate treatment. Another possible reason is that the diabetic patient is considered more vulnerable and that this disease has been experienced as a relative contraindication to some of these treatment modalities. Nevertheless, evidence-based coronary care treatment, including early coronary angiography and, if possible, revascularization, is at least as effective in the diabetic patient as in the non-diabetic patient and there are no indications for an increased propensity to side-effects, as shown in some recent studies.[261,327]

Risk stratification

Patients with ACS and concomitant DM, already known or newly recognized, are at high risk for subsequent complications. An extended risk assessment is important to identify specific threats and outline goals for the long-term management strategy. Early risk assessment is recommended, in order to identify possible co-morbidities and factors increasing the cardiovascular risk.[415,416] This includes: (i) a thorough evaluation of history and signs of peripheral, renal, and cerebrovascular disease; (ii) a careful evaluation of such risk factors as blood lipids, blood pressure, and of smoking and life style habits;[417,418] (iii) evaluation of clinical risk predictors including heart failure, hypotension, and risk for arrhythmia, with special focus on autonomic dysfunction; (iv) investigations of inducible ischaemia by means of ST-segment monitoring, exercise testing, stress echocardiography, or myocardial scintigraphy (whatever method is appropriate for the individual patient and clinical setting); (v) assessment of myocardial viability and LV function by means of echo-Doppler and/or magnetic resonance imaging. The reliability (sensitivity/specificity) of exercise testing, stress echocardiography, or myocardial scintigraphy is of a particular concern for detection of ischaemia in diabetic patients. Confounders are a potentially high threshold for pain due to autonomic dysfunction, the multivessel nature of the coronary disease, baseline electrocardiographic abnormalities, a commonly poor exercise performance of diabetic patients, coexistence of peripheral artery disease, and use of multiple medications. In this context, a careful clinical evaluation and focused evaluation of laboratory outcomes are of particular importance.

Recommendation

Early risk stratification should be part of the evaluation of the diabetic patient after ACS. Class IIa, Level of Evidence C.

Treatment targets

The management of diabetic patients following ACS aims at preventing further events, i.e. death, recurrent MI, progression to irreversible myocardial damage, or other cardiovascular events. Available treatment options, meant to preserve

Table 17 DM and total mortality in patients after the acute phase of ACS

Trial (Reference no.)	Inclusion	ACS diagnosis	Study type	*n* (diabetes)	Follow-up	Total mortality DM vs. no DM	RR-adjusted (CI)
MONICA-Augsburg[397]	1985–1992	Incident Q-wave MI	Register	2210 (468)	30 days	12.6% vs. 7.3%	
					5 years[a]	43.2% vs. 11.5%	1.64 (1.4–1.95)
Rotterdam[398]	1988–1989	UAP		282 (NA)	1, 3, 5, 7 years	6, 12, 19, 24% (in DM only)	1.89 (1.07–3.36)
The Onset Study[399]	1989–1993	AMI	Register 36% thrombolysis	1935 (399)	3.7 years	29% vs. 13%	1.7 (1.3–2.1) (F 2.7, M 1.3)
Zwolle[400]	1990–1995	STEMI	RCT SK or primary PCI	395 (74)	7.5 years	~48% vs. ~28% (*Figure 1*)	2.3 (1.5–3.5)
Sahlgrenska[401]	1988–1998	nonQ MI UAP	Register	4341 (722)	30 day	13% vs. 7.5%	1.6 (1.4–1.9)
					1 year	33.7% vs. 20.2%	
GUSTO-I[402]	1990–1993	STEMI	RCT thrombolysis	41021 (5944)	30 day	10.5% vs. 6.2%	1.77 (not reported)
					1 year	14.5% vs. 8.9%	(not reported)
GUSTO-IIb[403]	1994–1995	STEMI	RCT	12142 (2175)	30 days		
		NSTEMI			STEMI	6.9% vs. 4.1%	1.75 (1.5–2.1)
		UAP			NSTEMI	8.4% vs. 5.5%	1.34 (1.2–1.6)
					UA	6.2% vs. 3.3%	1.94 (1.5–2.5)
MITRA[404]	1994–2000	STEMI, discharged		8206 (1489)	17 months	19.1% vs. 10.4%	1.50 (1.3–1.8)
OASIS[391]	1995–1996	NSTEMI or UAP	Register	8013 (1718)	2 years	18% vs. 10%	1.56 (1.4–1.8) (F 1.98; M 1.28)
RIKS-HIA[388]	1995–1998	First AMI	Register	25633 (5193)	1 year	Not reported for all	1.48 (1.3–1.6)
						M: 22.3% vs. 13.0%	1.92 (1.7–2.1)
						F: 26.1% vs. 14.4%	2.10 (1.9–2.4)
FRISC-II[405]	1996–1998	NSTEMI or UAP	RCT; Invasive revascularization ± dalteparin 3 m	2158 (299)	2 years		
					Non-invasive	12.5% vs. 2.7%	NA
					Invasive	7.7% vs. 1.4%	5.43 (2.1–14.1)
VALIANT[406]	1999–2001	HF within 5 days since AMI	RCT: Valsartan Captopril	14703 (known DM 3400; new DM 580)	1 year	Known DM	1.43 (1.3–1.6)
						New DM	1.50 (1.2–1.9)

ACS = acute coronary syndromes; UAP = unstable angina pectoris; NA = not available; RCT = randomized controlled trial; SK = streptokinase; DM = diabetes mellitus; HF = heart failure; F = females; M = males; m = months

[a]5-year mortality rates for survivors over 28 days.

and optimize myocardial function, achieve stabilization of vulnerable plaques, prevent recurrent events by controlling prothrombotic activity, and to counteract progression of atherosclerotic lesions are summarized in *Table 18*.[417,418]

Evidence-based recommendation for secondary prevention is in general terms valid for patients with, as well as without, diabetes. Since diabetes is associated with consistently higher absolute risk for cardiovascular morbidity and mortality, the management strategy should, if anything, be even more ambitious in this category of patients. For an equal risk reduction, the number of patients needed to treat to save one life or prevent one defined endpoint is lower among diabetic patients due to the higher absolute risk. Important treatment targets are outlined in *Table 19*, summarizing recommendations for secondary prevention, based on accumulated evidence, including data from recent guidelines and consensus documents.[130,419-421]

Recommendation
Treatment targets, as listed in *Table 19*, should be outlined and applied in each diabetic patient following an ACS. Class IIa, Level of Evidence C.

Specific treatment

Thrombolysis

The most important goal of treatment in AMI is to restore patency in the compromised coronary artery. This can either be done with thrombolytic drugs or by mechanical intervention. Thrombolysis is at least as beneficial in diabetic as in non-diabetic patients. A meta-analysis of 43 343 MI patients, 10% of whom had a history of diabetes, revealed that the number of lives saved by thrombolytic therapy was 37 per 1000 treated patients in the diabetic cohort, compared with 15 among those without DM.[422] Thus, due to their higher risk, fewer numbers are needed to treat to save one life in the diabetic cohort, corresponding to a greater absolute benefit for thrombolytic treatment in diabetics than in non-diabetic patients. It is a myth that thrombolysis is contraindicated in diabetic patients due to an increased risk of eye or cerebral bleeding.

Recommendation
Patients with AMI and diabetes should be considered for thrombolytic therapy on the same grounds as their non-diabetic counterparts. Class IIa, Level of Evidence A.

Early revascularization

Revascularization is undertaken to counteract myocardial ischaemia, protect viable myocardium, and prevent progression to MI or death. The choice between percutaneous coronary intervention (PCI) and coronary artery bypass grafting (CABG) is discussed further in this chapter.

A recent meta-analysis of randomized trials including patients with non-ST-elevation myocardial infarction (NSTEMI) and unstable angina, revealed that an early invasive strategy, including coronary angiography followed by coronary revascularization, reduced mortality from 4.9 to 3.8% (OR 0.76; CI: 0.62–0.94), the composite of death or MI from 11.0 to 7.4% (OR 0.64; CI 0.56–0.75), and resulted in a 33% reduction of severe angina and re-hospitalizations during an average follow-up of 17 months, when compared to a strategy with an invasive approach only in case of inducible ischaemia or recurrent symptoms.[423]

Revascularization within 14 days following an AMI, ST-elevation as well as non-ST-elevation, caused a 53% reduction in 1-year mortality in patients without and 64%

Table 18 Treatment options based on accumulated evidence

Revascularization
Anti-ischaemic medication
Antiplatelet agents
Anti-thrombin agents
Secondary prevention by means of
Life style habits including food and physical activity
Smoking cessation
Blocking the RAS
Blood pressure control
Lipid-lowering medication
Blood glucose control

Table 19 Recommended treatment targets for patients with diabetes and CAD (adapted after the European Guidelines for Cardiovascular Disease Prevention)[419]

Blood pressure (systolic/diastolic; mm Hg)	<130/80
In case of renal impairment, proteinuria >1 g/24 h	<125/75
Glycaemic control	
HbA_{1c} (%)[a]	≤6.5
Glucose expressed as venous plasma mmol/L (mg/dL)	
Fasting	<6.0 (108)
Post-prandial (peak)	<7.5 (135) diabetes type 2 7.5–9.0 (135–160) diabetes type 1
Lipid profile expressed in mmol/L (mg/dL)	
Total cholesterol	<4.5 (175)
LDL-cholesterol	≤1.8 (70)
HDL-cholesterol	
Men	>1.0 (40)
Women	>1.2 (>46)
Triglycerides[b]	<1.7 (<150)
TC/HDL[b]	<3
Smoking cessation	Obligatory
Regular physical activity (min/day)	>30–45
Weight control	
BMI (kg/m^2)	<25
In case of overweight weight reduction (%)	10
Waist (optimum; ethnic specific; cm)	
Men	<94
Women	<80
Dietary habits	
Salt intake (g/day)	<6
Fat intake (% of dietary energy)	
Saturated	<10
Trans fat	<2
Polyunsaturated n-6	4–8
Polyunsaturated n-3	2 g/day of linolenic acid and 200 mg/day of very long chain fatty acid

[a]DCCT-standardized, for recalculation formula for some national standards in Europe.[156]
[b]Not recommended for guiding treatment, but recommended for metabolic/risk assessment.

among those with diabetes (15 vs. 5%; RR 0.36; CI 0.22–0.61).[424,425] The early invasive reperfusion strategy among diabetic patients with unstable angina or NSTEMI in the FRISC-II Trial resulted in significant reduction of the composite endpoint of death or myocardial re-infarction from 29.9–20.6% (OR 0.61; CI 0.36–0.54).[405] The relative impact of the early invasive strategy was of the same magnitude in both diabetic and non-diabetic patients. This means that, due to the significantly higher absolute risk, the relative benefit was substantially larger in diabetic than in non-diabetic patients. The NNT to save one death or MI was 11 for diabetic and 32 in non-diabetic patients.

Recommendation
Whenever possible, patients with diabetes and ACS should be offered early angiography and mechanical revascularization. Class IIa, Level of Evidence B.

Anti-ischaemic medication

Beta-blockade

Post-myocardial treatment with beta-blockade results in a general mortality reduction, as reflected by a systematic overview of scientific reports published 1966–1997 by Freemantle *et al.*[426] In this meta-analysis, the overall mortality reduction was 23% (CI 15–31%), which can be translated into a number of 42 patients needed to treat during 2 years to save one life.[426] BBs are particularly effective in decreasing post-infarction mortality and new infarcts in patients with a history of DM.[427–432]

Based on accumulated evidence of improved survival, prevention of re-infarction, and sudden cardiac death and reduction or treatment of late ventricular arrhythmias, oral BBs are, in the absence of contraindications, recommended for all diabetic patients with ACS.[417,418,433] Furthermore, such patients are more prone to develop heart failure and recent trials have clearly documented the beneficial effects of beta-blockade in heart failure patients.[541,543,544] Accordingly, although to a large extent based on subgroup analyses, a liberal use of BBs in diabetic patients with MI is advocated,[433,434] since the beneficial effects have a solid basis in pathophysiology. It seems reasonable to make individualized drug choices among different BBs bearing in mind concomitant conditions and type of diabetes treatment. Selective beta-1 antagonists may be preferred in case of insulin treatment and alfa-1-beta-adrenergic antagonists such as carvedilol may offer additional benefits for patients with peripheral artery disease or substantial insulin resistance.[434] Still, contemporary data report that diabetic patients with CAD are deprived from this life-saving treatment.[394,397,404] BBs should be used in adequate dosages as detailed in a recent expert consensus document on beta-adrenergic blockers.[433]

Recommendation
BBs reduce morbidity and mortality in patients with diabetes and ACS. Class IIa, Level of Evidence B.

Other drugs

Nitrates and calcium antagonists belong to anti-ischaemic drugs. Recent meta-analyses do not reveal survival benefits for any of them, although favourable effects have been reported for diltiazem in patients with non-ST-elevation infarctions.[418,435] Long-acting calcium channel antagonists and nitrates are therefore not generally recommended, but they may be of value for symptomatic relief in patients already treated with BBs or with contraindications for their use.[417,418]

Anti-platelet and anti-thrombotic agents

Reducing platelet aggregation by acetylsalicylic acid therapy is a cheap and effective way to reduce mortality and morbidity in patients with CAD, not the least those with ACS. It has been claimed, but not verified, that diabetic patients need particularly high doses of aspirin for efficient suppression of platelet-derived thromboxane A2. A systematic analysis of 195 trials including more than 135 000 patients (4961 with diabetes) at high risk for arterial disease given antiplatelet therapy in the form of aspirin, clopidogrel, dipyridamol, and glycoprotein IIb/IIIa antagonists (separately or in combination) revealed that the risk of stroke, MI, or vascular death was reduced by about 25%.[436] The benefits experienced among diabetic patients were somewhat lower. The anti-thrombotic trialists concluded that the optimal effective aspirin dose is 75–150 mg daily, with a loading dose of 150–300 mg to be introduced when an immediate effect is needed.

Recommendation
Aspirin should be given for the same indications and in similar dosages to diabetic and non-diabetic patients. Class II, Level of Evidence B.

When added to aspirin, the effect of thienopiridines (Ticlopidine, Clopidogrel), which block the adenosine diphosphate (ADP) receptor-dependent platelet activation, is favourable in patients with unstable angina and non-ST-elevation infarctions, lowering the incidence of cardiovascular death, MI, or stroke from 11.4 to 9.3%; (RR 0.80; CI 0.72–0.90).[437] The outcome of the CURE Trial resulted in the recommendation to use clopidogrel (75 mg daily) combined with aspirin (75–100 mg daily) to be continued for 9–12 months following an acute coronary event.[418,438] Among patients with diabetes and vascular disease, clopidogrel provides better protection from serious events (vascular death, re-infarctions, stroke, or recurrent hospitalization for ischaemia) than aspirin (RR 0.87; CI 0.77–0.88; CAPRIE).[439,440]

Recommendation
ADP receptor-dependent platelet activation (clopidogrel) should be considered in diabetic patients with ACS in addition to aspirin. Class IIa, Level of Evidence C.

ACE-inhibitors

Blockers of the RAS (ACE-inhibitors) have not been shown to offer any particular advantage in diabetic compared with non-diabetic patients in connection to an MI, except from a report from the GISSI-3 Trial. In a subgroup analysis from this study, early institution of lisinopril reduced mortality in patients with diabetes, however, not in their non-diabetic counterparts.[441] The possibility that the ACE-inhibitor ramipril may prevent cardiovascular events in people with diabetes was tested in the Heart Outcomes Prevention Evaluation (HOPE) Study. A total of 3654 patients with diabetes and previous CVD or one or more risk factors for such disease were recruited to a subgroup in which diabetes was a pre-specified study question.[373] There was a 25% reduction in the composite endpoint of MI, stroke, or

cardiovascular death, and a clear reduction in each of the component outcomes. More recently, the EUropean Trial on Reduction Of cardiac events with Perindopril in stable CAD (EUROPA) Study extended these findings to a population that in absolute terms had at lower cardiovascular risk than in HOPE. Reduction of cardiovascular morbidity and mortality with perindopril was observed irrespective of a relatively high use of other secondary prevention therapies. The proportionate benefit for patients with diabetes was similar to those in the overall population. The absolute benefit was, however, greater because of the higher event rate among diabetic subjects.[442,443]

Due to hypertension, more than 50% of patients with type 2 diabetes are exposed to a considerably increased cardiovascular risk. Strict control of the elevated blood pressure is an effective secondary preventive measure in these patients. Details on blood pressure control and the use of various drugs, including ACE-inhibitors, alone or in combination, is given elsewhere in these guidelines (see section on hypertension).

Recommendation
The addition of an ACE-inhibitor to other effective therapies reduces the risk for cardiovascular events in patients with diabetes and established cardiovascular disease. Class I, Level of Evidence A.

Lipid-lowering drugs

The use of lipid-lowering therapy is discussed in a separate chapter of these guidelines (see section on dyslipidemia).

Metabolic support and control

There are several reasons why intensive metabolic control during an AMI should be of benefit. It would direct myocardial metabolism from beta-oxidation of FFA towards less energy consuming glucose utilization. One way to achieve this effect is to infuse insulin and glucose. Intense insulin-based glucose control treatment also has the potential to improve platelet function, correct the disturbed lipoprotein pattern, and decrease PAI-1 activity, thereby improving spontaneous fibrinolysis (see section on pathophysiology). The concept of acute and/or chronic metabolic control was tested in the two DIGAMI trials. DIGAMI 1 recruited 620 patients with diabetes and AMI to be randomly assigned to serve as a control group or to a group receiving intensive insulin treatment, initiated by an insulin-glucose infusion during the first 24 h after MI.[323] One-year mortality was reduced by 30% in the intensively treated group, and this therapy tended to favourably influence all cardiovascular causes of death.[325] In a long-term follow-up over an average of 3.4 years, there was an 11% absolute mortality reduction in the group subjected to intense insulin treatment, implying one saved life for every nine patients treated.[409] Of particular interest was that patients without previous insulin treatment and at a relatively low risk benefited the most. HbA_{1c}, used as the measure of improved metabolic control, decreased on an average by 1.4% in this group of patients. An interesting finding was that the well-established epidemiological relationship between admission glucose level and mortality was only seen among the control patients, indicating that proper metabolic treatment in the peri-infarction period attenuated the harmful effect of a high blood glucose level on admission.[323]

The second DIGAMI Trial compared the three management protocols: acute insulin-glucose infusion followed by insulin-based long-term glucose control; insulin-glucose infusion, followed by standard glucose control; routine metabolic management according to local practice, in 1253 patients with type 2 diabetes and suspected AMI.[326] The DIGAMI 2 Trial did not show that an acutely introduced, long-term intensive insulin treatment strategy improves survival in type 2 diabetic patients following MI and did not demonstrate that initiating treatment with an insulin-glucose infusion is superior to conventional management. The overall mortality in DIGAMI 2 was, however, lower than expected. Moreover, glucose control was better than in DIGAMI 1 already at the onset of treatment and the three glucose management strategies did not result in a significantly different glucose control. Indeed, target glucose levels were not reached in the intensive insulin group and were better than expected in the two other arms. Blood glucose was fairly well controlled in DIGAMI 2 even if the targeted levels could not be reached in the insulin arm. Given a similar degree of glucose regulation, it seemed as if insulin *per se* did not improve the prognosis more than any other combination of glucose-lowering drugs. The DIGAMI 2 Trial clearly confirmed that glucose level is a strong, independent predictor of long-term mortality following MI in patients with type 2 diabetes, with a 20% increase in long-term mortality for an increase in updated plasma glucose by 3 mmol/L.

A meta-analysis of several early studies on glucose-insulin-potassium (GIK) therapy in AMI, including 1932 predominantly non-diabetic patients, suggested a proportional acute mortality reduction of 28%. The treatment effect was further enhanced if only studies utilizing high-dose intravenous GIK regimens were taken into consideration.[410] In the Estudios Cardiologicos Latinoamerica (ECLA) Trial, involving 400 patients, there was a trend towards a non-significant reduction in major and minor in-hospital events in patients allocated to GIK therapy. However, in a subgroup comprising 252 patients who also had reperfusion therapy, there was a significant reduction in mortality in the treated group compared with the controls.[411] The recent CREATE-ECLA Trial randomized more than 20 000 patients with acute ST-elevation infarction, out of whom 18% had type 2 diabetes, to high dose GIK or to standard care. This included acute reperfusion therapy in more than 80% of the patients. The overall outcome of this large-scale study was that GIK did not influence mortality, cardiac arrest, or cardiogenic shock.[444] It must be emphasized that none of these trials targeted a pure diabetic population or aimed at normalization of blood glucose *per se*. In fact, there was a significant increase in blood glucose levels in the CREATE-ECLA Trial, which may have contributed to the neutral result. The very consistent results from this trial strongly suggest that acute metabolic intervention by means of GIK has no place in the contemporary treatment of patients with AMI, if not used to normalize blood glucose.

In contrast, and as discussed in detail elsewhere in these guidelines (see section on intensive care), a Belgian surgical ICU Study which targeted a 'normal' glucose level (4.5–6.1 mmol/L; 80–110 mg/dL) in the actively treated group showed a significant decrease in mortality and also infection rate, compared with the conventional treated group.[445]

Based on present knowledge, there is reasonable evidence to initiate glucose control by means of insulin infusion in diabetic patients who are admitted for AMIs with significantly elevated blood glucose levels in order to reach normoglycaemia as soon as possible. Patients admitted with relatively normal glucose levels may be handled with oral glucose-lowering agents. In the follow-up, both epidemiological data and recent trials support that continued strict glucose control is beneficial. The therapeutic regime to accomplish this goal may include diet, life styles strategies, oral agents, and insulin (see also section on life style and comprehensive management). Since there is no definite answer to which pharmacological treatment is the best choice, the final decision can be based on decisions by the physician-in-charge in collaboration with the patient. Most importantly, the effect on long-term glucose control has to be followed and the levels should be targeted to be as normal as possible. Several outcome studies with novel agents or regimens are ongoing and will report in the near future.

Recommendation
Diabetic patients with AMI benefit from tight glucometabolic control. This may be accomplished by different treatment strategies. Class IIa, Level of Evidence B.

A comprehensive therapeutic strategy

Effective management of diabetic patients during and after acute coronary events is demanding, as the reduction of several risk factors including blood glucose must be achieved for satisfactory results.[71,446] An aggressive multi-disciplinary and multi-factorial therapeutic strategy is, however, beneficial. In the STENO 2 Study, such approach halved the risk of cardiovascular events in diabetic patients at high risk verified by concomitant microalbuminuria.[309] In the second DIGAMI Trial, aggressive treatment from the early onset of MI was the probable reason for the unexpectedly low, 18%, 2-year mortality, an outcome approaching that for patients with MI but free from diabetes.[326]

Revascularization (intervention by surgery or PCI angioplasty)

Table of Recommendations:

Recommendation	Class[a]	Level[b]
Treatment decisions regarding revascularization in patients with diabetes should favour CABG surgery over percutaneous intervention	IIa	A
Whenever possible, patients with diabetes undergoing coronary bypass surgery should be offered at least one and often multiple arterial grafts	I	C
Glycoprotein IIb/IIIa inhibitors are indicated in elective PCI in a diabetic patient	I	B
When PCI with stent implantation is performed in a diabetic patient, drug-eluting stents (DES) should be used	IIa	B
Mechanical reperfusion by means of primary PCI is the revascularization mode of choice in a diabetic patient with AMI	I	A

[a]Class of recommendation.
[b]Level of evidence.

Diabetes and coronary revascularization

Revascularization of narrowed or occluded coronary vessels by means of CABG was first introduced by thoracic surgeons in 1964[447,448] The less invasive revascularization modality, PCI, was introduced in 1977, rapidly growing to a new field in cardiology.[449] Many diabetic patients, who were previously considered candidates for surgery, have been successfully treated by PCI, with good long-term results. Revascularization procedures may be indicated in diabetic patients with stable or unstable coronary syndromes, covering the whole spectrum of ischaemic heart disease from asymptomatic patients to ST-elevation MI, ACS, and resuscitated sudden cardiac death. Patients with diabetes have a higher mortality and morbidity after CABG compared with non-diabetics, but this is also seen in patients undergoing PCI.[450-457] The influence of glucometabolic control on the outcome after revascularization (insulin vs. oral agents) is still unclear. Patients who require insulin have more adverse events, but this may be induced by a more advanced diabetes related morbidity, including atherosclerosis and/or microvascular disease, and perhaps also by so far unknown variables in these patients.[458-462]

CABG in diabetic compared with non-diabetic patients results in lower short- and long-term survival and more complications, including increased incidence of mediastinitis and sternal wound infections and delayed healing in general.[450,453,458,463-467] Bilateral mammary artery grafting may be a risk factor for complications in the presence of diabetes, but internal mammary artery grafts also improve long-term outcome.[451,459,463,466,467] Likewise, diabetic patients undergoing PCIs have a lower survival than their non-diabetic counterparts. They are at increased risk for adverse short- and long-term outcomes, including a higher need for in-hospital CABG, and a higher incidence of stent thrombosis, restenosis, demand of repeat revascularization and MI.[453,455-457,468,469]

Surgery vs. percutaneous intervention

The effectiveness of PCI and bypass surgery as a mode of revascularization has been compared in several RCTs, among them the Bypass Angioplasty Revascularization Investigation (BARI), Coronary Angioplasty vs. Bypass Revascularization Investigation (CABRI), Emory Angioplasty vs. Surgery Trial (EAST), and Randomized Intervention Treatment of Angina (RITA).[458,459,470-473]

In these studies, balloon angioplasty was applied as a mode of PCI and no coronary stents were used. Later, when stents became available, studies were conducted, comparing this new percutaneous technology with CABG in multivessel CAD.[474-477]

Major concerns were raised when a *post-hoc* subgroup analysis of BARI patients with diabetes and multivessel disease demonstrated a less favourable prognosis among those treated with PCI than those subjected to CABG (*Table 20*).[458,478] In BARI, the 7-year survival for the total population was 84.4% for surgically treated patients and 80.9% for PCI ($P = 0.043$). The corresponding proportions for diabetes patients were 76.4 vs. 55.7% ($P = 0.001$).

This suggests that the non-significant treatment difference between the two groups was limited to the PCI patients with diabetes. Furthermore, in BARI, the survival difference

was in fact also limited to those diabetic patients who received at least one arterial internal mammary graft.[459] These grafts are known to provide better long-term patency than saphenous vein grafts. BARI was not designed to focus on diabetic patients. The suspicion raised by BARI, that long-term prognosis after PCI might be worse in patients with diabetes with multivessel disease, was, however, confirmed by another large registry of consecutive revascularization procedures.[479]

Unrandomized patients, eligible for the BARI Study, were included in a registry. Their mode of revascularization was left to the discretion of patients and physicians. In this BARI-registry similar differences in mortality were not observed[456,460] (*Table 20*). In addition, three other studies, conducted in the balloon angioplasty era, could not confirm the conclusion from BARI with regard to diabetic patients undergoing PCI: RITA-1, CABRI, and EAST (*Table 20*).[471-473]

The Angina with Extreme Serious Operative Mortality Evaluation (AWESOME) Trial randomized only patients with unstable angina and high surgical risk. A total of 54% of the patients in the PCI group received stents and 11% received glycoprotein IIb/IIIa antagonists.[477] The combined impression from these studies is that survival does not differ, but that diabetic patients have a significantly higher incidence of repeat revascularization and that restenosis is still a major problem especially in this patient category (*Tables 20* and *21*).

Recommendation

Treatment decisions regarding revascularization in patients with diabetes should favour CABG surgery over percutaneous intervention. Class IIa, Level of Evidence A.

Whenever possible, patients with diabetes undergoing coronary bypass surgery should be offered at least one and often multiple arterial grafts. Class I, Level of Evidence C.

Adjunctive therapy

All studies mentioned still raise the question whether revascularization by means of PCI or CABG is to be preferred in patients with diabetes and multivessel disease.

Stents and later DES have been hailed to improve the outcome of PCIs in the diabetic patient. Although the results are promising, only one small study did in fact address subacute stent thrombosis, restenosis, and long-term outcome in this patient category, and other available data relate to subsets of patients included in studies on stents and DES.[457,462,480-482] A recent meta-analysis comparing DES with bare metal stents in diabetic subpopulations in several clinical trials revealed that DES were associated with an 80% RRR for restenosis during the first year of follow-up.[483] Future clinical trials comparing DES with coronary bypass surgery are certainly needed to determine the optimal revascularization strategy in diabetic patients with multivessel disease. Newer stents with improved design and especially new bare metal stents with thin struts have also been successful in decreasing the incidence of restenosis.[481] However, definite conclusions with regard to treatment outcome can only be drawn from a randomized comparison of these newer stents with DES in well-defined patients with diabetes.

Potent platelet glycoprotein IIb/IIIa inhibitors improve the outcome after PCIs when administered during the procedure in diabetic patients. In three randomized trials with abciximab, there was a 44% reduction of mortality after 1 year, suggesting that these agents are indicated in all diabetic patients undergoing PCI.[482] ADP receptor antagonists (thienopyridines), like clopidogrel, may prevent early as well as late thrombotic complications after stent implantation, particularly in patients with diabetes.[438]

Cardiac surgery has also witnessed major improvements over the past decade and new techniques like minimal invasive surgery, robot techniques, and off-pump procedures are promising with regard to short- and long-term outcome.[484] A recent paper renewed the interest in differences in revascularization techniques in multivessel disease, but whether this also applies to diabetic patients remains uncertain.[485,486]

In patients with diabetes, the progressive nature of the atherosclerotic disease, the marked endothelial dysfunction, and platelet and coagulation abnormalities are responsible for a less favourable outcome after

Table 20 Trials addressing diabetes and revascularization for multivessel disease

Trial (Reference no.)	Patients (no.)	Follow-up (years)	Mortality CABG (%)	Mortality PCI (%)	*P*-value
BARI[458]	353	7	23.6	44.3	<0.001
CABRI[471]	124	4	12.5	22.6	NS
EAST[472]	59	8	24.5	39.9	NS
BARI-registry[460]	339	5	14.9	14.4	0.73

Table 21 Revascularization in diabetes patients with multivessel disease in the stent-era

Trial (Reference no.)	Patients (no.)	Follow-up (years)	Mortality CABG (%)	Mortality PCI (%)	Revascularization CABG (%)	Revascularization PCI (%)	Mortality *P*-value
ARTS[474]	208	3	4.2	7.1	8.4	41.1	0.39
SoS[476]	150	1	0.8	2.5			NS
AWESOME[477]	144	5	34	26			0.27

ARTS, the Arterial Revascularization Therapy Study; SoS, the Stent or Surgery Trial.

revascularization. Additional treatment should be focused on these specific disease entities, with special attention paid to concomitant disease and risk factors like hypertension, dyslipidaemia, smoking, and life style (see other parts of these guidelines). However, no randomized trials have been conducted to see whether these measures will affect the outcome after revascularization procedures. Results from randomized trials and registries may be used to treat patients with diabetes with ACE-inhibitors or BBs after revascularization, but no randomized data are available.[372,456] Furthermore, no data are available regarding whether improved glycaemic control and pharmacological intervention can reduce the incidence of restenosis after PCI or improve patency of bypass grafts after CABG.[487-489]

Whether diabetes in general is associated with an increased physician's preference for either medical or revascularization treatment was recently addressed in the Euro Heart Survey on coronary revascularization. In a broad range of European practices, diabetes was not among the factors that determine treatment decisions in stable coronary disease.[490] However, the higher incidence of repeat revascularization in PCI-treated patients should always be taken into consideration. Although patients presenting with ACS have different clinical characteristics than those who present with stable coronary syndromes, the general opinion is that the approach with regard to the mode of revascularization has to be identical.[491]

Recommendation
Glycoprotein IIb/IIIa inhibitors are indicated in elective PCI in patients with diabetes. Class I, Level of Evidence B.

When PCI with stent implantation is performed in patients with diabetes, DES should be used. Class IIa, Level of Evidence B.

Revascularization and reperfusion in MI

Patients with diabetes or hyperglycaemia may have a different response to several treatment strategies used for MI.[400,492-494] In patients with ST-segment elevation MI, thrombolysis seems to be less effective in those with diabetes.[495] In general, increasing evidence suggests that primary PCI is preferable to thrombolysis as reperfusion therapy for ST-segment elevation MI.[496-498] Whether this benefit is present in patients with diabetes is less clear. Still, primary PCI has been suggested as the treatment of choice in high-risk patients, among whom are the diabetic patients.[496,497] Although thrombolysis is less beneficial in diabetic patients, revascularization and reperfusion by primary PCI may also be less successful, due to more diffuse CAD, smaller reference diameters, and a tendency for higher restenosis rates.[499,500] Patients with DM have an adverse prognosis after ST-segment elevation MI and myocardial reperfusion as assessed by ST-segment resolution and myocardial blush grade, demonstrating more frequently reduced blush and incomplete ST-segment resolution after primary angioplasty, compared with patients without diabetes.[400]

Identifying the optimal method of reperfusion in diabetic patients is of great clinical importance, as the number of ST-segment elevation MI patients with diabetes is high and their prognosis poor.[395,501] A recent analysis of diabetic patients included in 11 randomized trials demonstrated a survival benefit for those treated with primary PCIs over those with thrombolytic treatment.[497,498] These findings have been confirmed by two other studies.[502,503]

Cardiac surgery in the setting of ST-segment elevation MI is only indicated when the coronary anatomy is not suitable for a percutaneous intervention, after such intervention has failed and the area of myocardium at risk is large, or when mechanical complications occur.

Recommendation
Mechanical reperfusion by means of primary PCI is the revascularization mode of choice in diabetic patients with AMI. Class I, Level of Evidence A.

Unresolved issues

In patients with diabetes and CAD, both PCIs and CABG are treatment options, although it remains to be determined if one is preferable over the other. Reperfusion and revascularization in diabetic patients with ST-segment elevation MI should probably be accomplished by means of primary PCIs to ensure optimal reperfusion of the epicardial vessel, as well as on the level of the myocardial tissue.

Many previous randomized trials addressing these issues were performed before the current era of modern stent technology and new pharmacologic adjunctive therapy. Furthermore, the vast majority of studies only includes subgroups of patients with diabetes and was not dedicated to patients with diabetes in particular. Only new controlled randomized trials applying modern revascularization technology in patients with diabetes will give the answer whether CABG, hybrid revascularization procedures, or PCIs is the preferred treatment modality in these patients. Diffuseness of atherosclerotic involvement, type of diabetes, suitability for percutaneous intervention, clinical presentation, presence of chronic total occlusion, lesion morphology and involvement of proximal left anterior descending coronary artery, co-morbidity, and other factors may define subgroups that may benefit specifically from one or the other revascularization option. Other conditions like LV function, valvular abnormalities, and age may also be of crucial importance in making decisions.

In the Future Revascularization Evaluation in Patients with Diabetes Mellitus: Optimal Management of Multivessel Disease (FREEDOM) Trial, patients with diabetes will be randomized for CABG or PCIs with sirolimus-coated DES with the composite of death, MI, and repeat revascularization as the primary endpoint.[504] The Bypass Angioplasty Revascularization Investigation II-Diabetes (BARI-IID) Trial will enrol patients for PCIs or CABG and different regimens of optimal medical management, whereas the Coronary Artery Revascularization in diabetes Trial (CARDia) will enrol 600 diabetic patients to compare PCIs and CABG on a non-inferiority design base.[505] The 1500-patient, prospective, multicentre, multinational (European and North American), randomized SYNTAX Study[192] will enrol consecutive patients with *de novo* three-vessel disease and/or left main disease, randomizing patients for PCIs or CABG. Randomized patients will further be stratified on presence of diabetes. The primary endpoint

for this comparison is non-inferiority of major adverse cardiac and cerebral events between the two groups at 1 year. Until such trials addressing the key issues in revascularization in diabetic patients have been completed, an indicative classification is highly speculative.

Heart failure and diabetes

Table of Recommendations:

Recommendation	Class[a]	Level[b]
ACE-inhibitors are recommended as first-line therapy in diabetic patients with reduced LV dysfunction with or without symptoms of heart failure	I	C
Angiotensin-II-receptor blockers have similar effects in heart failure as ACE-inhibitors and can be used as an alternative or even as added treatment to ACE-inhibitors	I	C
BBs in the form of metoprolol, bisoprolol, and carvedilol are recommended as first-line therapy in diabetic patients with heart failure	I	C
Diuretics, in particular loop diuretics, are important for symptomatic treatment of diabetic patients with fluid overload due to heart failure	IIa	C
Aldosterone antagonists may be added to ACE-inhibitors, BBs, and diuretics in diabetic patients with severe heart failure	IIb	C

[a]Class of recommendation.
[b]Level of evidence.

Background

According to the guidelines issued by the ESC, the diagnosis of heart failure should be based on a combination of clinical symptoms of heart failure and signs of myocardial dysfunction. In clinical practice, heart failure is commonly divided into systolic dysfunction, representing an impaired capacity to eject blood from the left ventricle and diastolic dysfunction, an impaired ventricular filling due to relaxation abnormalities.[506]

Echocardiography is the preferred method for documentation of cardiac dysfunction and the most important measurement for diagnosing impaired systolic dysfunction is the LV ejection fraction (EF). Evidence of abnormal LV relaxation, diastolic distensibility, or diastolic stiffness are echocardiographic signs of diastolic dysfunction diagnosed in the presence of normal or mildly abnormal LV systolic function.[506] Echocardiography, including Tissue Doppler Imaging is useful in detecting myocardial dysfunction in diabetic patients, as well as, in the non-diabetic population.[507] Plasma concentrations of natriuretic peptides or their precursors may also be helpful for diagnosing heart failure in diabetic patients.[506,508]

The leading causes of chronic heart failure are hypertension and ischaemic heart disease.[506,509] Other common factors influencing the occurrence of this syndrome are smoking, overweight, physical inactivity, and type 2 diabetes, as well as, poor glucometabolic control observed as high FPG and elevated HbA_{1c}.[296,510-513]

Epidemiological aspects

Prevalence of heart failure and glucose abnormalities

The prevalence of heart failure varies somewhat in different studies. The prevalence of heart failure has been estimated to be 0.6–6.2% in Swedish men and this increases with age. This is similar to the overall prevalence of heart failure among both genders in the Rotterdam population and the Reykjavík Study.[514-516]

Considerably less is known about the prevalence of the combination of diabetes and heart failure. The most recent and extensive data on the prevalence of diabetes and heart failure are from the Reykjavík Study, showing that the prevalence of the combination heart failure and diabetes is 0.5% in men and 0.4% in women, increasing with increasing age. Heart failure was found in 12% of those with diabetes compared with only 3% in individuals without diabetes. Thus, there was a strong association between diabetes and heart failure.[516]

Incidence of heart failure and glucose abnormalities

Among British outpatients, the incidence of heart failure has been reported to be around 4/1000 person-years, rising with age. Similar data have been reported from Finland.[517,518]

Less information is available on the incidence of the combination of diabetes and heart failure. In the Framingham Study, the incidence of heart failure was double among males and five times higher in females with diabetes during 18 years of follow-up, compared with patients free from diabetes[519] and in a general population of elderly Italians the incidence of diabetes was 9.6% per year in heart failure patients.[520]

Prognostic implications

In the presence of diabetes and heart failure, the prognosis becomes deleterious.[521] Diabetes is also a serious prognostic factor for cardiovascular mortality in patients with LV dysfunction due to ischaemic heart disease.[522] In a general population in Reykjavík, the survival decreased significantly with the concomitant presence of both heart failure and glucose abnormalities, even after adjustment for cardiovascular risk factors and ischaemic heart disease.[523] This may be seen as an indicator of the serious implication of the combination of diabetes and heart failure.

Treatment

There are very few, if any, clinical trials on heart failure treatment specifically addressing the diabetic patients. Information on treatment efficacy of various drugs is therefore based on diabetic subgroups included in various heart failure trials. A disadvantage of this is that the subgroups are not always well defined as regards the diabetic state and treatment. Most data favour a proportionately similar efficacy in patients with and without diabetes. Traditional treatment of heart failure in diabetic patients is currently based on diuretics, ACE-inhibitors, and BBs, as outlined in other guidelines.[420,506] Moreover, it is assumed that meticulous metabolic control should be beneficial in heart failure patients with diabetes.[524]

ACE-inhibitors

The use of ACE-inhibitors is indicated both in asymptomatic myocardial dysfunction and symptomatic heart failure, since

they improve the symptoms and reduce mortality. ACE-inhibitors are beneficial in moderate to severe heart failure (*Table 22*) with and without diabetes.[525-529] Diabetics represent a rather large subgroup of the patient cohorts in several important heart failure trials.

The Studies of LV Dysfunction (SOLVD) Study showed a similar effect of enalapril treatment in patients with compromised LV function, with and without diabetes[530] and in the Assessment of Treatment with Lisinopril and Survival (ATLAS) Trial, the mortality reduction was at least as good in the diabetic as in the non-diabetic group when comparing high and low doses of lisinopril.[531] The third Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico (GISSI 3) and the Survival and Ventricular Enlargement (SAVE) Trials have shown beneficial effects on morbidity and mortality of ACE-inhibitor treatment in diabetic patients after MI.[441,532,533]

Hypoglycaemia has been reported following the institution of ACE-inhibitors in patients with diabetes on glucose-lowering treatment.[534,535] It is therefore recommended to monitor plasma glucose carefully in the early phase of the institution of an ACE-inhibitor in such patients.

Recommendation
ACE-inhibitors are recommended as first-line therapy in diabetic patients with reduced LV dysfunction with or without symptoms of heart failure. Class I, Level of Evidence C.

Angiotensin-II-receptor blockers
Angiotensin receptor blockers can be used as an alternative to ACE-inhibitors to improve morbidity and mortality in heart failure patients or even in combination with ACE-inhibitor in symptomatic heart failure patients.[506] The use of angiotensin receptor blockers has not been tested primarily in patients with heart failure and diabetes, but in subgroup analysis of large clinical trials the beneficial effects were equivalent to that of ACE-inhibitors.[536-538]

Recommendation
Angiotensin-II-receptor blockers have similar effects in heart failure as ACE-inhibitors and can be used as an alternative or even as added treatment to ACE-inhibitors. Class I, Level of Evidence C.

Beta-blockers
Beta-blockade decreases myocardial free fatty acid exposure, thereby changing that metabolic pathway in type 2 diabetes.[539,540] There are no studies that specifically address the use of beta-blockade in patients with diabetes and heart failure. Subgroup analysis of diabetic patients in large heart failure trials has, however, shown that BBs reduce mortality and improve symptoms in moderate to severe heart failure to a similar extent in patients with and without diabetes. Since mortality is considerably higher among diabetic than non-diabetic heart failure patients, the number needed to treat to save one life is substantially less in the diabetic cohort. The following BBs may, based on the outcome of clinical trials including subgroups of patients with diabetes, be recommended as first-line treatment in patients with heart failure and diabetes: Metoprolol (MERIT-HF), Bisoprolol (CIBIS II), Carvedilol (COPERNICUS and COMET).[432,541-545]

Recommendation
BBs in the form of metoprolol, bisoprolol, and carvedilol are recommended as first-line therapy in diabetic patients with heart failure. Class I, Level of Evidence C.

Diuretics
Diuretics are mandatory for relief of symptoms that are due to fluid overload. These drugs should, however, not be used in excess since they induce neuro-hormonal activation.[506] Although no studies specifically look into the outcome of the use of diuretics in a heart failure population consisting of diabetic patients, loop diuretics, rather than diuretics which may impair the glucometabolic state further, are recommended.[546]

Recommendation
Diuretics, in particular loop diuretics are important for symptomatic treatment of patients with fluid overload due to heart failure. Class IIa, Level of evidence C.

Aldosterone antagonists
The addition of aldosterone antagonists is indicated in severe forms of heart failure and may then improve longevity.[547] No specific information is, however, available from clinical trials on the administration of aldosterone antagonists in patients with diabetes and heart failure. The institution of blockers of the renin-angiotensin-aldosterone system should be made with caution and surveillance of kidney function and potassium, since nephropathy is not infrequent among patients with diabetes and heart failure.

Recommendation
Aldosterone antagonists may be added to ACE-inhibitors, BBs, and diuretics in diabetic patients with severe heart failure. Class IIb, Level of Evidence C.

Table 22 The effect of ACE-inhibitor treatment in major heart failure trials

Trial (Reference no.)	Number of participants	Diabetes (%)	Outcome
CONSENSUS[528]	253	18	31% reduction of 1-year mortality
SAVE[532]	2 231	22	19% all-cause mortality risk reduction, 21% risk reduction of cardiovascular morbidity
ATLAS[531]	3 164	19	14% mortality risk reduction with high-dose ACE-inhibitor treatment
GISSI 3[441]	18 131	15	30% reduction in mortality after 6 weeks

Glucose lowering treatment and metabolic modulation

Insulin

The main effect of insulin is to decrease blood glucose but it may also increase myocardial blood flow, decrease heart rate, and cause a modest increase in cardiac output.[548,549] Insulin treatment in patients with diabetes and heart failure is under debate. It has been shown to have beneficial effects on the myocardial function, but also to be associated with increased mortality.[540,550] Further studies are needed to establish the specific role of insulin treatment beyond the role as an anti-diabetic agent in patients with diabetes and heart failure. In general, it is assumed that meticulous metabolic control would be beneficial in heart failure patients with diabetes,[524] but this hypothesis has not yet been tested in prospective clinical trials.

Thiazolidinediones

Thiazolidinediones are insulin sensitizers that are used as glucose-lowering drugs in the treatment of diabetes. Because of a risk for fluid retention, and thereby worsening of heart failure symptoms, the use of these drugs are considered contraindicated in heart failure patients in New York Heart Association Class III–IV.[552] They may, however, if needed, be attempted in patients with milder degrees of heart failure, New York Heart Association Class I–II.

Metabolic modulators

Drugs, such as trimetazidine, etomoxir, and dichloroacetate, which aim to shift myocardial metabolism from oxidation of FFA towards glycolysis, have been tested in patients with myocardial dysfunction and diabetes, but their usefulness has not been demonstrated.[553–556]

Arrhythmias: AF and sudden death

Table of Recommendations:

Recommendation	Class[a]	Level[b]
Aspirin and anticoagulant use as recommended for patients with AF should be rigorously applied in diabetic patients with AF to prevent stroke	I	C
Chronic oral anticoagulant therapy in a dose adjusted to achieve a target international normalized ratio (INR) of 2 to 3 should be considered in diabetic patients with AF and one other moderate risk factor for thromboembolism, unless contraindicated	IIa	C
Control of glycaemia even in the pre-diabetic stage is important to prevent the development of the alterations that pre-dispose to sudden cardiac death	I	C
Microvascular disease and nephropathy are indicators of increased risk of sudden cardiac death in diabetic patients	IIa	B

[a]Class of recommendation.
[b]Level of evidence.

Diabetes, AF, and risk of stroke

Diabetes and AF

AF is the most common arrhythmia and one of the most important risk factors for stroke.[557] The prevalence of AF is estimated at 0.4% of the general population and it increases with age.[558] It is uncommon under 50 years of age, but it reaches a prevalence of ~10% in individuals over 80 years.[559] The incidence of AF is less than 0.1% per year in subjects under 40 years of age and increases to 1.5% per year in women and 2% in men over 80 years of age.[560] AF is more frequent in patients with structural heart disease, i.e. heart failure and valvular disease, but a significant proportion occurs in subjects with no history of CVD.

Diabetes is not infrequent in patients with AF. Among patients in the Etude en Activité Liberale sur le Fibrillation Auriculaire (ALFA) Study reporting on AF in patients seen in general practice, the proportion of diabetes in patients with chronic AF was 13.1%, making diabetes a common associated condition surpassed only by heart failure and hypertension.[561] Several cardiac and non-cardiac factors have been demonstrated to have an effect on the incidence of AF. The Manitoba Follow-up Study[562] estimated the age-specific incidence of AF in 3983 males, to identify risk factors for the development of this arrhythmia. Diabetes was significantly associated with AF with a relative risk of 1.82 in the univariate analysis. However, in the multivariate model the association with diabetes was not significant, suggesting that the increased risk of AF in diabetic men may depend on the presence of ischaemic heart disease, hypertension, or heart failure.

In the Framingham Heart Study,[563] 2090 men and 2641 women, free of a history of AF and between the ages of 55 and 94 years were studied to identify independent risk factors for the arrhythmia, including diabetes. During up to 38 years of follow-up, 264 men and 298 women developed AF. Diabetes was significantly associated with AF in both genders even after adjustment for age and other risk factors (OR, 1.4 for men and 1.6 for women). Although the mechanisms underlying this association remain to be elucidated, diabetes seems to favour the occurrence of AF.

Diabetes and risk of stroke in AF

The risk of ischaemic stroke is significantly increased among patients with AF.[570] The rate of ischaemic stroke among patients with AF included in primary prevention clinical trials and not treated with anti-thrombotic therapy averaged 4.5% per year,[565] which is nine times the proportion in patients without AF. The risk of stroke increases with age. As an example, the annual risk increased from 1.5% in subjects aged 50–59 years to 23.5% for those aged 80–89 years in the Framingham Study.[558,566]

In epidemiological studies, AF has been identified as a risk factor for stroke in both diabetic[567,568] and non-diabetic patients. The prevalence of diabetic patients varies from 8 to 34% in observational studies and in primary and secondary stroke prevention trials in patients with AF.[569–574]

The role of risk factors for stroke among patients with AF not receiving anticoagulants has been studied in subjects participating in several randomized trials of anti-thrombotic therapy. The AF Investigators (AFI) group[565] analysed the data from the pooled control groups of five primary prevention trials with warfarin or aspirin in patients with AF. The purpose of the analysis was to identify clinical features indicative of a high vs. low risk of stroke. At the time of randomization, 14% of patients had diabetes. Risk factors that predicted stroke in multivariate analyses of control patients were increasing age, history of hypertension, previous

transient ischaemic attack (TIA) or stroke, and diabetes. Specifically, a diagnosis of DM was an independent risk factor for stroke with a relative risk of 1.7.

The rate of embolic events originating from the atrium in patients with AF increases with the reduction of left atrial appendix flow velocity and the presence of echo contrast at transoesophageal ultrasound examination.[575] A relation between the number of additional risk factors in patients with AF, including diabetes, and the presence of echo contrast or reduced flow velocity in left atrial appendix has been demonstrated,[576] suggesting that factors like hypertension and diabetes may influence the complex thrombo-embolic mechanisms.

Anti-thrombotic therapy in AF

Anti-thrombotic therapy prevents stroke in patients with AF. A meta-analysis of 16 randomized clinical trials on 9874 patients was performed to characterize the efficacy of anticoagulant and antiplatelet agents for prevention of stroke in AF.[577] Oral anticoagulation was effective for primary and secondary prevention of stroke in studies comprising 2900 patients, with an overall 62% reduction of relative risk (95% CI: 48–72%). The ARR was 2.7% per year for primary prevention and 8.4% for secondary prevention. Major extracranial bleedings were increased by anticoagulant therapy by 0.3% per year. Aspirin reduced stroke by 22% (95% CI: 2–38%) with an ARR of 1.5% per year for primary prevention and 2.5% per year for secondary prevention. In five trials, comparing anticoagulant therapy with antiplatelet agents in 2837 patients, warfarin was more efficacious than aspirin with an RRR of 36% (95% CI: 14–52%). These effects were observed in both permanent and paroxysmal AF.

Although warfarin is superior to aspirin for reducing stroke in patients with AF, the absolute and RRR are determined by the initial risk for stroke.[577] High-risk patients, with stroke rates greater than 4% per year, show a larger RRR by oral anticoagulation compared with aspirin, whereas the RRRs are smaller in patients with lower stroke rates. Accordingly, oral anticoagulation is most beneficial for patients at higher risk for stroke, whereas the risks outweigh the benefit in patients at low risk. Thus, quantifying the risk of stroke is crucial for determining which AF patients would benefit most from anticoagulant therapy.

Diabetes and stroke risk stratification schemes

Different stroke risk stratification schemes have been proposed for patients with AF and in most of them diabetes is taken into consideration as an important risk factor for stroke. Patients are considered at low, moderate, and high risk of stroke in relation to age, previous stroke, or TIA and the presence of additional risk factors, such as hypertension, diabetes, CAD, and heart failure. However, the importance of diabetes as a risk factor for stroke differs among the stratification schemes. In the AFI scheme,[565] diabetic patients are considered at high risk, independent of age. In the American College of Chest Physicians (ACCP) scheme, they are classified at moderate risk and high risk only if another risk factor is present,[578] whereas diabetes is not included as a risk factor in the Stroke Prevention in AF III Study (SPAF) scheme.[579] Two recently developed schemes are based on scores: the CHADS2 (acronym derived from the individual stroke risk factors: Congestive heart failure, Hypertension, Age >75 years, Diabetes, prior Stroke, or TIA)[580] and the Framingham scheme.[581] In CHADS2, two points were given for prior stroke or TIA (hence, the '2'), and one point was assigned for each of the other factors. In the Framingham scheme, a point system based on age (0–10 points), gender (6 points for female; 0 for male), blood pressure (0–4 points), DM (4 points), and prior stroke or TIA (6 points) was developed. A prospective cohort study tested the predictive accuracy of these five-stroke risk stratification schemes by pooling individual data from 2580 participants with non-valvular AF, who were prescribed aspirin in five multicentre trials on anti-thrombotic therapy.[582] All schemes predicted stroke, but the number of patients categorized as low- and high-risk varied substantially. AF patients with prior cerebral ischaemia were classified as high risk by all five schemes and low-risk patients were also identified by all schemes. However, only CHADS2 successfully identified primary prevention patients who were at high risk of stroke. Of note is that the presence of diabetes is an important contributor in the risk stratification of this scheme. In the 2006 guidelines on AF from the American College of Cardiology/American Heart Association/European Society of Cardiology (ACC/AHA/ESC) task force,[583] diabetes is classified as a moderate risk factor together with age >75 years, hypertension, heart failure, and a LVEF < 35%.

Anti-thrombotic therapy in diabetic patients

Both the AHA/ACC/ESC guidelines for AF[583] and the American College of Chest Physicians[584] recommend anti-thrombotic therapy for all patients with AF, apart for those with contraindications. The choice of anti-thrombotic agent should be based on the relative risk and benefit for the individual patient, considering the absolute risk for stroke and bleeding with various treatment modalities. In patients with permanent or paroxysmal AF who already had a stroke or a TIA, anticoagulant therapy with an INR between 2.0 and 3.0 is indicated, independently of age or the presence of additional risk factors. Also patients with more than one moderate risk factor for thrombo-embolism, whereof diabetes is one, should receive anticoagulant therapy. In patients considered to be at increased risk for bleeding (e.g. >75 years of age) but without clear contraindications to oral anticoagulation, a lower INR target of 2.0 (range 1.6–2.5), may be considered.

Recommendations for anti-thrombotic therapy in AF in the presence of only one moderate risk factor is, according to the 2006 AHA/ACC/ESC guidelines, aspirin 81–325 mg daily or anticoagulant therapy. Aspirin is indicated in a dose of 325 mg daily as an alternative in patients with contraindications to oral anticoagulation. In all patients with AF in whom anticoagulant therapy is indicated, INR should be determined at least weekly at the beginning of therapy and monthly when the patient is stable.

Overall and although data from multicentre randomized studies investigating the role of anticoagulants or aspirin in the prevention of stroke in patients with diabetes and AF are not available, it seems appropriate to conclude that diabetes is a risk factor for stroke and that this should be taken into account in the decision on appropriate therapy.

Recommendation
Aspirin and anticoagulant use as recommended for patients with AF should be rigorously applied in diabetic patients with AF to prevent stroke. Class I, Level of Evidence C.

Chronic oral anticoagulant therapy in a dose adjusted to achieve a target INR of 2 to 3 should be considered in diabetic patients with AF and one other moderate risk factor for thromboembolism, unless contraindicated. Class IIa, Level of Evidence C.

Sudden cardiac death

Epidemiology of sudden cardiac death in diabetes

Sudden cardiac death is a major cause of mortality in the Western population, with ischaemic heart disease as the most important substrate. The presence of co-morbidities identifies the subgroup of patients who are at a high risk.[585] Diabetes is a marker of adverse prognosis in patients after an MI. Although there are no doubts on the excess of total mortality of patients with diabetes after MI, more debate surrounds the issue of whether diabetes increases sudden cardiac death and conflicting results are present in the literature.

Some methodological considerations have to be made before entering in the evaluation of the evidence present in the medical literature.[585] Sudden death is a very difficult end-point to be assessed in clinical trials because of several methodological reasons. First of all, the definition of sudden cardiac death may vary substantially from one study to another, additionally the modality of death (sudden or not sudden) may be 'arbitrary' especially when death is unwitnessed and finally the methodology used to define the cause of death (autopsy vs. death certificate vs. whatever information is available) may also determine important differences in the percentage of death labelled as sudden cardiac.[585] When investigating the link between diabetes and sudden cardiac death, the methodological difficulties doubles as also the definition of glucose intolerance/diabetes may vary among different studies, thereby affecting the proportion of 'diabetic' patients present in various studies. Having made these considerations, the presence of discrepancies between results in the different studies that have investigated the role of diabetes as a risk factor for sudden cardiac death will appear less surprising. Interestingly, however, it appears that studies with large series of patients with very long follow-up (>20 years) support the existence of a positive association between diabetes and sudden cardiac death.

The Framingham Study was one of the first sources of long-term follow-up investigating risk factors for sudden cardiac death in the population. Diabetes was associated with an increased risk of sudden cardiac death in all ages (almost four-fold) and the sudden death risk ratios associated with diabetes were consistently greater in women than men.[586] The importance of diabetes as a risk factor for sudden cardiac death in women was recently investigated by data from the Nurses' Health Study,[587] which included 121 701 women aged 30 to 55 followed for 22 years. It was reported that sudden cardiac death occurred as the first sign of heart disease in 69% of cases, even if almost all the women who died suddenly had at least one cardiac risk factor. Diabetes was a very strong risk factor, as it was associated with almost a three-fold increased risk of sudden death, as compared with hypertension that was associated with a 2.5-fold increased risk and obesity with a 1.6-fold increased risk. These data confirm that diabetes is a strong risk factor for sudden cardiac death in both genders. Interestingly, data are also available to demonstrate that diabetes increases the relative risk for sudden cardiac death in different ethnic groups. The Honolulu Heart Programme[588] investigated the role of diabetes as a pre-disposing factor in middle aged Japanese-American men followed for 23 years. This study showed an increased relative risk for sudden cardiac death in subjects with diabetes and glucose intolerance, as compared with the non-diabetic individuals. It was concluded that prevention of diabetes should be regarded as a measure to reduce sudden cardiac death, not only because diabetes predisposes to MI, but also because it increases risk for arrhythmic death. More recently, the investigators of the Paris Prospective Study[589] demonstrated that, the risk of sudden cardiac death, but not that of fatal MI, was increased in patients with diabetes as compared with those without. Similarly, the Group Health Cooperative[590] presented a large study including 5840 individuals and reinforced the view that diabetes is a strong risk factor for sudden cardiac death in a French population. It seems logical to conclude that most of the evidence concurs to support the concept that diabetes is a risk factor for sudden cardiac death.

Pathophysiology of SCD in diabetes

Diabetic patients have a higher incidence of cardiac arrhythmias, including ventricular fibrillation and sudden death. The causes underlying the increased vulnerability of the electrical substrate in these patients are unclear and it is likely to be the consequence of the interplay of several concomitant factors. (i) Atherosclerosis[591] and (ii) microvascular disease[295] are increased in patients with diabetes and they concur to the development of ischaemia that pre-disposes to cardiac arrhythmias. (iii) Diabetic autonomic neuropathy[592,593] leads to abnormal reflexes and innervation of the diabetic heart influencing electrical instability. (iv) The electrocardiogram of diabetic patients presents repolarization abnormalities manifesting as prolonged QT interval and altered T waves[593] that may reflect abnormal potassium currents.[595] It seems therefore likely that factors like CAD, direct metabolic alterations, ion channel abnormalities, and autonomic dysfunction may all contribute to create the substrate for sudden cardiac death in the diabetic heart. However, since Ewing *et al.*[596] proposed that diabetic neuropathy may be a major determinant of electrical vulnerability of the heart in diabetes, several lines of research were activated which supported the view that altered heart rate variability,[597] alteration of QTc interval,[598] and abnormal respiration[599] were all arrhythmogenic consequences of abnormal cardiac innervation and that diabetic neuropathy indeed is a key link between diabetes and excess of sudden cardiac death. Most of these investigations were, however, single-centre studies lacking prospective evaluation or large and representative cohorts of patients. Their aim was to show that autonomic neuropathy was a risk factor for sudden cardiac death. They did not engage in the evaluation of whether it remained an independent risk factor for arrhythmic

death when adjusting for different covariates. Accordingly, the role of autonomic neuropathy as a major determinant of sudden cardiac death has recently been questioned and novel approaches to the problem have emerged from large observational studies.

In a study by Jouven *et al.*,[590] the investigators moved away from the evaluation of the risk of sudden cardiac death in 'diabetic' vs. 'non-diabetic' patients instead focusing on the relative risk of sudden cardiac death in groups of patients with different values of glycaemia. The study showed, that the higher the values of glycaemia, the higher the risk of SCD. Following adjustment for age, smoking habits, systolic blood pressure, heart disease, and glucose-lowering treatment, even patients with borderline diabetes defined as non-fasting glycaemia between 7.7 and 11.1 mmol/L (140 and 200 mg/dL), had an increased risk of sudden cardiac death (OR 1.24 compared with patients with normoglycaemia). The presence of microvascular disease, defined as retinopathy or proteinuria, and female gender increased the risk of sudden cardiac death in all groups. This study importantly emphasizes that glucose intolerance seems to be a continuous variable directly related to the risk of sudden cardiac death, rather than supporting the previous view of risk being related to a specific threshold of glucose intolerance as suggested by the 'dichotomous' approach of comparison between 'diabetic' vs. 'non-diabetic' patients. This fits with the present concept that cardiovascular risk increases well below present thresholds for diabetes and at glucose levels that usually have been considered fairly normal.[64]

The Framingham Investigators[600] studied the influence of glucose levels on heart rate variability in a large community-based population. They demonstrated that, after adjusting for covariates, indexes of reduced heart rate variability were influenced by plasma glucose. High glycaemic levels were followed by a lower heart rate variability. Similar findings were reported by the Atherosclerotic Risk in Community Study (ARIC),[601] that showed that even the pre-diabetic patients already have abnormalities of autonomic cardiac function and altered heart rate variability. These two studies confirmed that glucose levels should be considered as a continuous variable influencing the autonomic control of the heart. Unfortunately, these studies were not designed to answer the question whether reduced heart rate variability in diabetic patients is an independent predictor of sudden cardiac death. At present, this pressing question remains unanswered.

The Rochester Diabetic Neuropathy Study[602] was designed to define the risk factors for sudden cardiac death and the role of diabetic autonomic neuropathy in a population of 462 diabetic patients followed for 15 years. In a univariate analysis, many covariates were statistically associated with sudden cardiac death including older age, HDL cholesterol, nephropathy stage, creatinine, microalbuminuria and proteinuria, previous MI, prolonged corrected QT, bundle branch block, and a composite autonomic severity score, among several others. Interestingly, necropsy findings demonstrated that all victims of sudden cardiac death had signs of coronary artery or myocardial disease and a bivariate analysis showed that autonomic dysfunction QTc and HDL lost their significant association with sudden cardiac death after adjusting for nephropathy. Overall, the data from this study suggest that kidney dysfunction and atherosclerotic heart disease are the most important determinants of the risk of sudden cardiac death while neither autonomic neuropathy nor QTc are independent predictors of the risk for sudden cardiac death. Unfortunately, this study did not include heart rate variability among the parameters introduced in multivariate analysis. Thus, robust data assessing the value of heart rate variability as an independent predictor of sudden cardiac death in diabetic patients are still lacking.

Based on available evidence, it seems that:

(1) Glucose intolerance, even at a pre-diabetic stage, is associated with progressive development of a variety of abnormalities that adversely affect survival and predispose to sudden cardiac death.
(2) The identification of independent predictors of sudden cardiac death in diabetic patients has not yet progressed to a stage where it is possible to devise a risk stratification scheme for the prevention of such deaths in diabetic patients.
(3) In a single study, microvascular disease and nephropathy have been identified as indicators of increased risk of sudden cardiac death in diabetic patients.

Recommendations

Control of glycaemia even in the pre-diabetic stage is important to prevent the development of the alterations that pre-dispose to sudden cardiac death. Class I, Level of Evidence C.

Microvascular disease and nephropathy are indicators of increased risk of sudden cardiac death in diabetic patients. Class IIa, Level of Evidence B.

Peripheral and cerebrovascular disease

Peripheral vascular disease

Table of Recommendations:

Recommendation	Class[a]	Level[b]
All patients with type 2 diabetes and cardiovascular disease are recommended treatment with low-dose aspirin.	IIa	B
In diabetic patients with peripheral vascular disease treatment with clopidogrel or low molecular weight heparin may be considered in certain cases.	IIb	B
Patients with critical limb ischaemia should, if possible, undergo revascularization procedures.	I	B
An alternative treatment for patients with critical limb ischaemia, not suited for revascularization, is prostacyclin infusion.	I	A

[a]Class of recommendation.
[b]Level of evidence.

Background

Subjects with diabetes have a two- to four-fold increase in the incidence of peripheral vascular disease and an abnormal ankle-brachial blood pressure index is present in ~15% of such patients.[603–605] The symptomatic manifestations of peripheral vascular disease are intermittent claudication and

critical limb ischaemia and both these conditions are increased in the diabetic population. Impairment of the circulation in the foot, due to diabetic macro and microvascular disease, is the most common non-traumatic reason for limb amputation. The prevalence of peripheral vascular disease increases with advancing age, duration of diabetes, and peripheral neuropathy. The latter condition may mask the symptoms of limb ischaemia and thus disease progression may be advanced before patients and healthcare providers realize that peripheral vascular disease is present.

Peripheral vascular disease is a marker of general atherosclerosis and patients with both symptomatic and asymptomatic peripheral vascular disease most often also have coronary and/or cerebrovascular disease.[603] Early diagnosis of peripheral vascular disease in diabetic patients is important for the prevention of progression of peripheral vascular disease as well as for prediction of overall cardiovascular risk. The vascular obstructions in subjects with diabetes are often located more distally than in non-diabetic subjects. Thus, the typical diabetic peripheral vascular disease is located in the popliteal artery or in the vessels of the lower leg.[606,607] The calcification of the media layer of the vessels is also a typical hallmark of diabetic peripheral vascular disease.[607,608]

Diagnosis

Symptoms of leg ischaemia in diabetic patients with peripheral neuropathy are often atypical and vague. Rather than experiencing pain in legs, the patient may suffer from leg fatigue or only inability to walk at a normal pace. Physical examination is of critical importance for the diagnosis. Palpation of pulses in the leg and visual inspection of the feet are essential. Dependent rubor, pallor when the foot is elevated, absence of hair growth, and dystrophic toenails are signs of peripheral ischaemia.

An objective measure of peripheral vascular disease is the ankle-brachial blood pressure index, defined as the ratio between the arterial pressure at the ankle level and in the brachial artery with the highest pressure. A Doppler device is used to record the pulse in the dorsal pedal artery or the posterior tibial artery, while decreasing pressure in a cuff placed at the ankle level (*Figure 15*). Measurement is made in the supine position after 5 min of rest. The reproducibility of this method is good and the ankle-brachial blood pressure index should normally be above 0.9. This measurement is valuable for early detection of peripheral artery disease and also for a better stratification of overall cardiovascular risk.

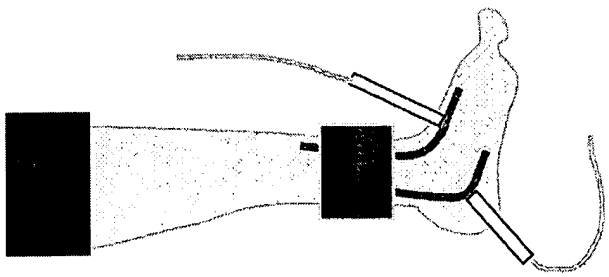


Figure 15 Measurement of blood pressure at the ankle level. A Doppler device is used to detect pulses in the posterior tibial artery and the dorsal pedal artery while slowly deflating the cuff around the ankle. The highest pressure recorded in the artery is the ankle pressure.

An ankle-brachial blood pressure index below 0.5 or an ankle pressure below 50 mm Hg is indicative of severely impaired circulation of the foot. An ankle-brachial blood pressure index above 1.3 indicates poorly compressible vessels as a result of stiff arterial walls, which usually in diabetic patients are due to atherosclerosis in the media layer of the arterial wall. In situations where a raised ankle-brachial blood pressure index is recorded or a false normal value is suspected, the blood pressure should also be measured at the level of the toe by a minicuff and a technique suitable for blood flow detection in the toe. A patient with critical limb ischaemia is defined as a patient with chronic ischaemic rest pain, ulcers, and gangrene attributable to objectively proven arterial disease.[609] It is important to consider that ulcers may often exist in the diabetic foot despite a normal macrocirculation. These ulcers are then due to disturbances in the microcirculation and most often also to neuropathy. Nevertheless, such ulcers must be dealt with in a meticulous fashion, since gangrene and amputation may result also from this condition.[610]

A thorough investigation, aiming at a detailed description of the anatomy of the vascular obstructions, should only be performed in patients in whom an invasive procedure to restore blood flow is indicated. The method of choice is duplex ultrasound. An arterial angiography should only be performed when it is likely that an invasive intervention to restore arterial circulation may be possible. Alternatives to duplex for determining the localization and degree of obstruction before angiography are segmental pressure measurements or oscillography. *Table 23* depicts the different methods for evaluating the peripheral circulation.

Treatment

General measures and platelet inhibition

For diabetic patients with peripheral vascular disease, general measures to reduce overall cardiovascular risk

Table 23 Investigations of the peripheral circulation in diabetic patients

At the physicians office in every patient
Inspection
Dependent rubor
Pallor with elevation
Absence of hair growth
Dystrophic toenails
Ulcers or gangrenes
Palpation
Pulses
Dry and cool skin
Sensibility
Pressure measurement
Ankle and arm blood pressure
At the vascular laboratory (when appropriate)
Distal and/or segmental pressure measurements
Oscillography
Treadmill testing (with or without distal pressure after exercise)
Duplex sonography
For evaluation of the microcirculation
Transcutaneous oxygen pressure
Vital capillaroscopy
At the radiology department
Magnetic resonance imaging
Angiography

should be intensive,[286,309,611] as it has been described in detail elsewhere in these guidelines (see section on life style and comprehensive management). Smoking cessation is mandatory and regular exercise is also important. Treatment of hypertension should be vigorous, but in patients with critical limb ischaemia and very low distal perfusion pressures it may be dangerous for the foot to lower blood pressure too much. The survival of tissues in the distal extremities must be prioritized until the critical situation resolves. In such cases, blood pressure should be kept at a level permitting adequate arterial inflow to the distal extremity.

Platelet inhibition with low-dose aspirin, in the magnitude of 75–250 mg/day, is indicated in all patients with type 2 diabetes and CVD who do not have a contraindication and for patients with severe peripheral vascular disease, further inhibition of platelet aggregation by clopidogrel or dipyridamole may be indicated in certain cases, along with anticoagulation with low molecular weight heparin as the first agent of choice.[611–614]

In patients with non-ischaemic neuropathic ulcers, it is of utmost importance to remove any external pressure from the ulcer area, sometimes necessitating immobilization of the patient in a wheelchair. These ulcers will then most often heal without any intervention directed towards improving the macrocirculation. Careful wound dressing and orthopaedic shoes or appropriate bandaging should be handled by a specialized clinic.[610] Unfortunately, many amputations have been performed in cases where careful conservative treatment would have saved the extremity.

Recommendation
All patients with type 2 diabetes and CVD are recommended treatment with low-dose aspirin. Class IIa, Level of Evidence B.

In diabetic patients with peripheral vascular disease, treatment with clopidogrel or low molecular weight heparin may be considered in certain cases. Class IIb, Level of Evidence B.

Revascularization
If anatomically possible, a revascularization procedure should be attempted in all patients with critical limb ischaemia.[609] This can be performed by means of a percutaneous transluminal angioplasty or as a surgical procedure, preferably a bypass with the saphenous vein as the conduit. Percutaneous transluminal angioplasty is the method of choice if short-segment stenoses occur in proximal segments above the knee. Proximal percutaneous transluminal angioplasty can be combined with a more distal bypass operation. Patients with intermittent claudication should be revascularized if they have disabling symptoms and proximal vessel disease.[609] For patients with claudication, who need a bypass to the lower leg vessels, a more conservative approach is indicated.

Recommendation
Patients with critical limb ischaemia, should if possible, undergo revascularization procedures. Class I, Level of Evidence B.

Medical treatment of critical limb ischaemia
The only pharmacological agent so far convincingly shown to have a positive influence on the prognosis of patients with critical limb ischaemia is a synthetic prostacyclin (Ilomedin, Iloprost), which is given intravenously daily for a period of 2–4 weeks. In a meta-analysis, rest pain and ulcer size improved in comparison with placebo. More importantly, the probability of being alive with both legs still intact after 6 months was 65% in the Iloprost-treated group, compared with 45% in the placebo-treated patients.[615]

Recommendation
An alternative treatment for patients with critical limb ischaemia not suited for revascularization is prostacyclin infusion. Class IIa, Level of Evidence A.

Stroke

Table of Recommendations:

Recommendation	Class[a]	Level[b]
Normalization of blood pressure is recommended in all patients with diabetes for the prevention of stroke	I	A
For stroke prevention, blood pressure lowering is more important than the choice of drug. Inhibition of the renin-angiotensin-aldosterone system may have additional benefits beyond blood pressure lowering *per se*	IIa	B
Inhibition of the renin-angiotensin-aldosterone system may be considered also in diabetic patients with normal blood pressure levels	IIa	B
Patients with stroke should be treated with statins according to the same principles as non-diabetic subjects with stroke	I	B
Antiplatelet therapy with aspirin is recommended for primary and secondary prevention of stroke	I	B
Patients with acute stroke and diabetes should be treated according to the same principles as stroke patients without diabetes	IIa	C
Optimization of metabolic conditions including glycaemic control should be aimed for	IIa	C

[a]Class of recommendation.
[b]Level of evidence.

Background

The relative risk for stroke is increased in subjects with diabetes by a factor of 2.5–4.1 for men and 3.6–5.8 for women.[76,616,617] After correction for other risk factors for stroke, which are more common in diabetic subjects, the risk still remains increased more than two-fold meaning that DM is a strong independent risk factor for stroke.[83,618] The relationship between hyperglycaemia *per se* and stroke is, however, much less clear than the relationship between hyperglycaemia and MI. Diabetic complications such as proteinuria, retinopathy, and autonomic neuropathy further increase the risk for stroke.[619,620]

The type of stroke is usually ischaemic and the ratio between ischaemic and haemorrhagic stroke is higher in diabetic subjects than in the general population.[621]

Further, diabetes is an independent risk factor for death from stroke.[87,622]

A TIA has been shown to predict the occurrence of a stroke within 90 days, thus underlining the severity of TIA especially in diabetic patients.[623]

Prevention of stroke

Measures to prevent stroke should include a multifactorial strategy[309] aimed at treatment of hypertension, hyperlipidaemia, microalbuminuria, hyperglycaemia, and the use of antiplatelet medication, as outlined elsewhere in these guidelines (see also section on hypertension). Results from the HOPE Study and Perindopril Protection Against Recurrent Stroke Study (PROGRESS) suggest that the reduction of stroke incidence in diabetic subjects during treatment based on ACE-inhibitors was greater than would be anticipated from the blood-pressure-lowering effect alone and the effect was also evident in normotensive individuals.[373,624] In the Losartan Intervention For Endpoint reduction in hypertension Study (LIFE) Trial the same trend was found with an angiotensin receptor blocker.[378] However, in several other trials, including Anti-hypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial (ALLHAT), there was no apparent benefit of one class of antihypertensive drug over another in this respect.[380,384]

Treatment with statins has been shown to reduce the incidence of stroke in high-risk patients, but the diabetic subpopulations in the trials have most often been too small to allow a reliable subgroup analysis. In the HPS, a sizeable subgroup of 5963 diabetic patients were randomized to placebo or 40 mg of simvastatin daily. Simvastatin reduced the incidence of stroke by 24%.[344]

Antiplatelet therapy has also been shown to reduce the incidence of stroke in diabetic patients and is indicated for both primary and secondary prevention of stroke.[625] Aspirin in a low dose (75–250 mg daily) should be the initial choice, but in case of intolerance clopidogrel 75 mg once daily should be given.[438,613] In patients with recurrent stroke, a combination of aspirin and dipyridamol should be considered.[626,627] The alternative combination with aspirin and clopidogrel seems less safe since it was associated with an increased risk of bleeding without any benefit in terms of cardiovascular outcome in the MATCH-Trial, performed in 7599 patients of whom 68% had diabetes.[628] Further, in the CHARISMA-Study, no benefit was evident from long-term dual antiplatelet therapy with aspirin and clopidogrel.[629] In patients with AF, anticoagulant therapy should be given for stroke prevention as is outlined in the section of arrhythmias–AF and sudden cardiac death.[630]

The high frequency of early stroke following TIA indicates investigation within 7 days of the index event to reduce the risk of a subsequent, and potentially more serious, neurological event. Evaluation with echocardiography and carotid ultrasound is indicated. An increase in cerebral microemboli is detectable by transcranial doppler (TCD) and high microembolic loads appear to be surrogate markers for future neurological events.[631] After a TIA or stroke caused by carotid-artery disease, medical treatments can be optimised in risk patients, avoiding the need for emergency carotid surgery allowing patients to undergo safer elective surgery.[632] Carotid endarterectomy for the prevention of stroke in patients with high-grade stenosis of the carotid artery has been shown to be effective, although it has not been specifically investigated in diabetic patients.[632] Since complications during and after this procedure are more frequent in diabetic as compared with non-diabetic subjects, special consideration should be paid to the overall risk for peri- and post-operative morbidity and mortality when deciding on surgical interventions in the patient with diabetes.[633] An alternative to endarterectomy, carotid artery angioplasty and stenting (CAS), which has been found to be at least not inferior to endarterectomy, may prove to be a preferable method in high risk patients.[634]

Recommendation

Normalization of blood pressure is recommended in all patients with diabetes for the prevention of stroke. Class I, Level of Evidence A.

For stroke prevention, blood pressure lowering is more important than the choice of drug. Inhibition of the renin-angiotensin-aldosterone system may have additional benefits beyond blood pressure lowering *per se*. Class IIa, Level of Evidence B.

Inhibition of the renin-angiotensin-aldosterone system may be considered also in diabetic patients with normal blood pressure levels. Class IIa, Level of Evidence B.

Patients with stroke should be treated with statins according to the same principles as non-diabetic subjects with stroke. Class I, Level of Evidence B.

Antiplatelet therapy with aspirin is recommended for primary and secondary prevention at stroke. Class I, Level of Evidence B.

Treatment of acute stroke

The treatment in the acute phase of stroke in diabetic patients should follow the same principles that govern the treatment of stroke in the general population. Thrombolysis is an effective treatment for ischaemic stroke if instituted within 3–4 h.[635] It reduces mortality and disability from stroke but is associated with a risk of haemorrhage and its use and effects in diabetes require further evaluation by registration in an existing quality registry (SITS-MOST: www.acutestroke.org).

Conservative treatment of stroke includes close surveillance of vital functions, optimization of circulation and metabolic conditions, including glycaemic control, in a stroke ward.[636] Patients should receive early neurological rehabilitation and correction of abnormalities as outlined above in the section of prevention of stroke. Recent studies suggest that early intervention against hypertension during the acute phase of stroke may be beneficial but currently it is recommended to acutely reduce only very high blood pressures, above 220 mm Hg systolic and/or 120 mm Hg diastolic, and then with great caution not lowering blood pressure to levels that may enhance ischaemia. Blood pressure should not be lowered by more than 25% during the first day of treatment.[637]

Recommendation

Patients with acute stroke and diabetes should be treated according to the same principles as stroke patients without diabetes. Class IIa, Level of Evidence C.

Optimization of metabolic conditions including glycaemic control should be considered as in any other acute disease condition. Class IIa, Level of Evidence C.

Intensive care

Table of Recommendations:

Recommendation	Class[a]	Level[b]
Strict blood glucose control with intensive insulin therapy improves mortality and morbidity of adult cardiac surgery patients	I	B
Strict blood glucose control with intensive insulin therapy improves mortality and morbidity of adult critically ill patients	I	A

[a]Class of recommendation.
[b]Level of evidence.

The evolution of intensive care medicine during the last three to four decades has fostered a tremendous increase in the immediate survival of patients suffering from previously lethal, acute insults. Hence, patients now frequently enter a chronic phase of critical illness during which they remain dependent on vital organ support for a more or less extended time period. Mortality has remained high among these prolonged critically ill patients with, on an average, a 20% risk of death. Non-resolving multiple organ failure is the most frequent cause of death and occurs regardless of the initial disease for which admission to the intensive care unit (ICU) was required. Post-operative glycaemic control with intensive insulin therapy in critically ill patients clearly proved to be beneficial with regard to mortality and morbidity of the patients, irrespective of previously diagnosed diabetes.[445] Since it is not clear yet whether glucose levels should also be controlled pre- or peri-operatively, we here focus on the impact of this therapy during the patient's stay in the ICU.

Hyperglycaemia and outcome of critical illness

The stress imposed by critical illness leads to the development of metabolic and endocrine abnormalities. The patients usually become hyperglycaemic, due to insulin resistance and accelerated glucose production, known as 'stress diabetes' or 'diabetes of injury'.[638,639] In the acute phase of critical illness, hepatic glucose production is enhanced by upregulation of both gluconeogenesis and glycogenolysis, although serum levels of insulin, which normally suppresses these pathways, are high. Increased levels of glucagon, cortisol, growth hormone, catecholamines, and cytokines all play a role.[640-645] Apart from stimulated glucose production, impaired peripheral insulin-mediated glucose uptake contributes to the hyperglycaemic state. The exact pathophysiology of hyperglycaemia during prolonged critical illness remains less clear.

For a long time, it had been accepted that stress-induced hyperglycaemia in critically ill patients is beneficial to organs that largely depend on glucose for their energy supply but do not require insulin for glucose uptake. However, several recent studies clearly identify hyperglycaemia as an important risk factor in terms of mortality and morbidity of these patients. A meta-analysis on patients with MI revealed a strong and consistent association between the development of stress hyperglycaemia and increased risk of in-hospital mortality, and congestive heart failure (CHF) or cardiogenic shock.[646] Even mild elevations of fasting glucose levels in patients with CAD undergoing PCI have been associated with a substantial mortality risk.[647] Furthermore, the glucose level of patients undergoing CABG appeared to be an important predictor of delayed extubation.[648] A retrospective analysis of a heterogeneous population of critically ill patients also revealed that even a modest degree of hyperglycaemia was associated with substantially increased hospital mortality.[649] Approximately 30% of these patients were admitted to the ICU for cardiac indications. Another report studying the occurrence of hyperglycaemia among critically ill children with widely varying pathology and of which 24% had undergone cardiovascular surgery, showed a correlation with higher in-hospital mortality and increased length of stay.[650] Similarly, hyperglycaemia predicted increased morbidity and mortality after stroke,[651] severe brain injury,[652,653] trauma,[654,655] and severe burn injury.[656]

Blood glucose control with intensive insulin therapy in critical illness

A landmark prospective, randomized, controlled study on a large group of patients admitted to the ICU predominantly after extensive surgery or for complications developing after extensive surgery revealed major clinical benefits of intensive insulin therapy during critical illness.[445] In the conventional insulin therapy group, only excessive hyperglycaemia above 11.9 mmol/L (215 mg/dL) was treated with insulin, aiming to keep concentrations between 10.0 and 11.1 mmol/L (180-200 mg/dL). This protocol resulted in mean blood glucose levels of around 8 to 9 mmol/L (150-160 mg/dL), i.e. hyperglycaemia. Insulin was administered to the patients in the intensive insulin therapy group to maintain blood glucose levels between 4.4 and 6.1 mmol/L (80-110 mg/dL) and resulted in mean blood glucose levels of around 5 to 6 mmol/L (90-100 mg/dL) i.e. normoglycaemia, without detectable risk of hypoglycaemia-induced adverse events. The baseline characteristics, including the admission glucose levels and the percentage of patients with previously diagnosed diabetes, were comparable for the two treatment groups. Tight blood glucose control with insulin strikingly lowered the mortality during the period in the ICU from 8.0 to 4.6% (43% reduction). This benefit was most pronounced among patients who required intensive care for more than 5 days, with an ICU mortality reduction from 20.2 to 10.6% and an in-hospital mortality reduction from 26.3 to 16.8%. More than 60% of the total patient population was included after cardiac surgery. In this subgroup, intensive insulin therapy reduced ICU mortality from 5.1 to 2.1%. Of the long-stay cohort of patients, approximately one-third was admitted to the ICU after cardiac surgery.

Besides saving lives, intensive insulin therapy largely prevented several critical illness-associated complications.[445] The incidence of critical illness polyneuropathy was reduced by 44%, the development of blood stream infections by 46%, and acute renal failure requiring dialysis or haemofiltration by 41%. The need for red blood cell transfusions was a median 50% lower, indicating that anaemia developed less frequently. Patients were also less dependent on prolonged mechanical ventilation and intensive care. The clinical benefits of this therapy were equally present in most diagnostic subgroups, including the cardiac patients. For the latter

subgroup, a follow-up study showed that intensive insulin therapy also improved long-term outcome, when given for at least a third day in ICU, with maintenance of the survival benefit up to 4 years after randomization.[657] Risk for hospital re-admission and dependency on medical care were similar in both groups. The short-term glycaemic control with insulin during intensive care did not induce a substantial burden for the patient, his/her relatives or society, although the perceived quality of social and family life appeared to be moderately compromised. Particularly in the patients with isolated brain injury, intensive insulin therapy protected the central and peripheral nervous system from secondary insults and improved long-term rehabilitation.[658]

Importantly, the Leuven protocol of glycaemic control in a predominantly surgical patient population[445] was recently proven, in a large RCT, to be similarly effective in a strictly medical ICU patient population.[659] In the intention-to-treat group of 1200 patients, morbidity was significantly reduced, with lower occurrence of newly developed kidney injury, earlier weaning from mechanical ventilation, and earlier discharge from the ICU and from the hospital. The latter patients also developed hyper-bilirubinaemia less frequently. There was no difference in bacteraemia or prolonged antibiotic therapy requirement but the number of long-stay patients with hyper-inflammation was reduced. In the intention-to-treat group, insulin therapy did not significantly alter mortality (in-hospital mortality from 40.0 to 37.3%, $P = 0.3$). This was not surprising, as the study was not powered for this mortality endpoint. In the target group of long-stay patients, defined as receiving at least a third day of intensive care for which the study was powered, intensive insulin therapy reduced in-hospital mortality from 52.5% in the conventional to 43.0% in the intensive insulin therapy group ($P = 0.009$) and reduced morbidity even more strikingly.

An earlier observational study also largely confirmed the clinical benefits of the surgical ICU Trial[445] in 'real-life' intensive care of a heterogeneous medical/surgical population.[660] In this study, the impact of implementing a tight glucose management protocol was documented, comparing the new outcome results with historical controls. Approximately 18% of the patients were referred to intensive care for a cardiac diagnosis. In this study, blood glucose control was somewhat less strict, as intensive insulin therapy aimed for glucose levels below 7.8 mmol/L (140 mg/dL) and intravenous insulin was only administered if glucose levels exceeded 11.1 mmol/L (200 mg/dL) on two successive measurements. In this way, mean glucose levels of 8.4 mmol/L (152 mg/dL) in the baseline period decreased to 7.3 mmol/L (131 mg/dL) in the protocol period. After the implementation of the protocol, hospital mortality was 29% lower, length of intensive care stay 11% lower, whereas 75% fewer patients developed new renal failure and 19% fewer required red blood cell transfusion. The prevention of severe infections could not be confirmed, but the incidence of this complication was already low in the baseline period. However, another prospective, randomized, controlled study, although small, revealed decreased incidence of nosocomial infections in a predominantly surgical ICU patient population.[661] In this study, intensive insulin therapy targeted glucose levels between 4.4 and 6.7 mmol/L (80–120 mg/dL), resulting in mean daily glucose levels of 6.9 mmol/L (125 mg/dL) vs. 9.9 mmol/L (179 mg/dL) in the standard glycaemic control group. In an observational study of patients with DM undergoing cardiac surgery, intravenous insulin infusion to eliminate hyperglycaemia also lowered in-hospital mortality compared with the historical control group, with fewer deep sternal wound infections and shorter length of hospital stay.[662]

Whereas it has clearly been demonstrated that tight glucose control is achievable and safe in post-operative and medical ICU patients, peri-operative glycaemic control during cardiopulmonary bypass appears to be much more difficult, at times unattainable, and carries a high risk of inducing post-operative hypoglycaemia.[663] However, another group was successful in reliably maintaining normal glucose levels during open heart surgery by using the hyperinsulinaemic normoglycaemic clamp technique.[664] A summary of different trials on intensive insulin therapy in critical illness is given in *Table 24*.

Recommendation

Strict blood glucose control with intensive insulin therapy improves mortality and morbidity of adult cardiac surgery patients. Class I, Level of Evidence B.

Table 24 Published trials on intensive insulin therapy in critical illness

Study reference	Van den Berghe *et al.*[445]	Van den Berghe *et al.*[659]	Krinsley[660]	Grey and Perdrizet[661]	Furnary *et al.*[662]
Patient population	Surgical	Medical	Surgical/medical	Surgical	Cardiac surgery in diabetic patients
Number of patients	1548	1200/767[a]	1600	61	4864
Randomized study	Yes	Yes	No	Yes	No
Target glucose	<6.1	<6.1	<7.8	<6.7	<8.3
Mortality	↓	↓	↓		↓
Critical illness polyneuropathy	↓				
Bacteraemia/severe infections	↓	–	–	↓	
Acute renal failure	↓	↓	↓		
Red blood cell transfusions	↓		↓		
Duration of mechanical ventilation	↓	↓			
Length of stay	↓	↓	↓		↓
Deep sternal wound infections					↓

[a]Morbidity effect in all intention-to-treat patients ($n = 1200$); morbidity and mortality effect in the patients that required at least a third day in ICU ($n = 767$).

Strict blood glucose control with intensive insulin therapy improves mortality and morbidity of adult critically ill patients. Class I, Level of Evidence A.

Mechanisms behind improved outcome with intensive insulin therapy

Multivariate logistic regression analysis indicated that hyperglycaemia and a high dose of insulin were associated with a high risk of death.[665] Hence, it was the blood glucose control, and/or other metabolic effects of insulin that accompany tight blood glucose control, and not the insulin dose administered *per se* that contributed to the improved survival with intensive insulin therapy. The association between high insulin dose and mortality is likely explained by more severe insulin resistance in the sicker patients who have a high risk of death. The risk of death indeed appeared to linearly correlate with the degree of hyperglycaemia, with no clear cut-off below which there was no further benefit.[665] Patients who received conventional insulin therapy and who developed only moderate hyperglycaemia (6.1–8.3 mmol/L or 110–150 mg/dL) had a lower risk of death than those with severe hyperglycaemia (8.3–11.1 mmol/L or 150–200 mg/dL), whereas they were at higher risk of death than patients whose blood glucose levels were controlled below 6.1 mmol/L (110 mg/dL) with intensive insulin therapy. Other data also suggest that the mortality benefits can be attributed to glycaemic/metabolic control, rather than the absolute insulin doses administered.[649,666] For the prevention of critical illness polyneuropathy, bacteraemia, anaemia, and acute renal failure, tight glycaemic control below 6.1 mmol/L (110 mg/dL) similarly appeared to be of crucial importance.[665]

If indeed avoiding hyperglycaemia is crucial, it appears striking that by doing so only for the relatively short period, during the patient's need for intensive care, this strategy prevented the most feared complications of critical illness. Normal cells protect themselves from moderate hyperglycaemia by downregulation of glucose transporters.[667] On the other hand, chronic hyperglycaemia causes complications in diabetic patients in a time frame which is several orders of magnitude longer than the time it took to prevent life-threatening complications during intensive care. Thus, hyperglycaemia appears more acutely toxic in critically ill patients than in healthy individuals or diabetic patients. Upregulation of insulin-independent glucose uptake, mediated by the glucose transporters GLUT-1, GLUT-2, or GLUT-3 and resulting in cellular glucose overload, may play a role.[668] Part of the improvement with intensive insulin therapy is therefore likely explained by preventing glucose toxicity to the mitochondrial compartment,[669] the endothelium,[670] the neurons,[658] and immune cells.[671] However, also other effects of insulin may contribute to improved outcome, including the partial correction of the abnormal serum lipid profile,[672] the prevention of excessive inflammation,[671,673] and counter-action of the catabolic state evoked by critical illness.[669,671] Although data from different studies appear controversial, administration of insulin in a GIK regimen has shown to improve myocardial function and to protect the myocardium during AMI, open heart surgery, endotoxic shock, and other critical conditions.[674,675] However, the recent large randomized CREATE-ECLA Trial on GIK infusion in patients with AMI and the DIGAMI-2 Trial in patients with diabetes and AMI failed to show an effect of this intervention on survival, cardiac arrest, and cardiogenic shock.[326,444] Although direct anti-apoptotic properties of insulin, independent of glucose uptake, and involving insulin signalling have been shown to play a role in the described cardioprotective action of insulin,[674,676,677] such effects may be counteracted by elevated levels of blood glucose.[674] As such, lack of glucose control in the CREATE-ECLA and the DIGAMI-2 Trial might explain the controversy surrounding GIK using different protocols.[326,444,678] Adequately designed studies are therefore needed to fully assess the efficacy of GIK for myocardial protection and to dissect the impact of glycaemic control from that of insulin.

Health economics and diabetes

Table of Recommendations:

Recommendation	Class[a]	Level[b]
Lipid-lowering provides a cost-effective way of preventing complications	I	A
Tight control of hypertension is cost-effective	I	A

[a]Class of recommendation.
[b]Level of evidence.

Cost-of-illness studies

The most widely used method to assess the burden of diabetes is through cost-of-illness studies, which strives to assess the total cost caused by a disease or condition.[679,680] A search for cost-of-illness studies in diabetes, published in the English language, was performed by searching Medline, Embase, and HEED (the Health Economics Evaluation Database). Most published studies were performed in the US,[681–688] although some studies investigated the situation in European countries. These are summarized in *Table 25*. With the recognition of diabetes as a global disease, there have been a number of studies published for other countries as well.[689–694]

Most cost-of-illness studies have not made a distinction between type 1 and type 2 diabetes. This is unfortunate, since the two types of the disease affect different age groups and require different management. Another difficulty when comparing the results from different studies is that different methodological approaches may give different results. This has several explanations: (i) there is uncertainty about the incidence and prevalence of the disease, (ii) studies use different methods for attributing costs, (iii) different cost items have been included, (iv) evaluation principles differ, (v) data includes measurement error, and (vi) there are different approaches to estimate the cost of diabetes, e.g. including all costs for patients with diabetes gives a higher estimate than including only the costs attributable to diabetes and its complications, which in turn gives a higher estimate than only including costs due to the diagnosis diabetes as such.[705]

The CODE 2 Study[706] was designed to measure the total healthcare costs for patients with type 2 diabetes in eight European countries using the same methodological

Table 25 Cost-of-illness studies of diabetes in Europe—an overview

Reference	Country and year	Diabetes type
Gerard *et al.* (1989)[695]	England and Wales, 1992	1 and 2
Gray *et al.* (1995)[696]	England and Wales, 1992	1
Henriksson and Jönsson (1998)[697]	Sweden, 1994	1 and 2
Henriksson *et al.* (2000)[698]	Sweden 1999	2
Jönsson (1983)[699]	Sweden, 1978	1 and 2
Kangas *et al.* (1993)[700]	Finland, 1989	1 and 2
Lucioni *et al.* (2003)[701]	Italy, 1998	2
Oliva *et al.* (2004)[702]	Spain, 2002	1 and 2
Spri (1997)[703]	Sweden, 1993	1 and 2
Triomphe *et al.* (1988)[704]	France, 1984	1 and 2

Table 26 Direct medical costs for patients with type 2 diabetes in eight European countries and percentage of healthcare expenditure in the respective countries (1998)[706]

Country	Total costs (Million Euro)	Cost per patient (Euro)	Percentage of healthcare expenditure
Belgium	1 094	3295	6.7
France	3 983	3064	3.2
Germany	12 438	3576	6.3
Italy	5 783	3346	7.4
The Netherlands	444	1889	1.6
Spain	1 958	1305	4.4
Sweden	736	2630	4.5
United Kingdom	2 608	2214	3.4
All countries	29 000	2895	5.0

approach. Patients from Belgium, France, Germany, Italy, the Netherlands, Spain, Sweden, and the UK were included. The study used a bottom-up, prevalence-based design, which means that all healthcare costs for diabetes patients were collected. Due to the strong impact of co-morbidity in type 2 diabetes patients, it is not possible to separate which resource use is due to diabetes and which is due to other diseases. This can only be done with epidemiological methods, comparing patients with and without diabetes. Efforts were made to ensure consistency in terms of data collection, analysis, and reporting of results, which means that this study gives an opportunity for international comparisons. *Table 26* shows the total cost per country, the cost per patient and the share of healthcare costs accounted for by patients with diabetes.

The total healthcare cost for patients with diabetes in the eight countries amounted to 29 billion Euro. Per capita cost varied from 1305 Euro per patient in Spain to 3576 Euro in Germany. In addition, the estimated share of total healthcare costs varied significantly between countries, indicating that despite striving for the same method of data collection, there may have been differences between how the study was conducted in different countries. The very low figure for the Netherlands may reflect lower costs, but more probably a selection bias in the patients studied and/or a too low estimate of the prevalence of type 2 diabetes. Differences in the definition of healthcare expenditures between countries may also be a factor to consider when analysing the differences between countries.

The cost of complications

The results from the CODE-2 Study show that the main cost-driver in diabetes is not the disease as such or the treatment of diabetes, but rather the complications caused by diabetes. In the study, patients were divided into complication-free, having microvascular complications only, having macrovascular complications only, or having both macro and microvascular complications. In these three groups, the relative costs were 1.7, 2.0, and 3.5 times higher than the costs among patients without complications.[707] The key driver of this increase in costs was a higher cost for hospitalization among patients with complications. This is natural, since patients are not frequently hospitalized for their diabetes, whereas macrovascular complications, such as MI, lead to immediate hospitalization. The total prevalence of macrovascular complications was 33%, with 12% of patients having a history of heart failure, 9% of MI, and 17% of the patients suffering angina pectoris.

Table 27 shows the distribution of the direct costs on different cost items. Hospitalizations are the largest cost component in the sample as a whole as well, once again indicating the importance of complications. It is interesting to note that cardiovascular drugs are the single most important category of drugs, accounting for about one-third of drug costs. This is more than the costs of insulin and oral diabetic drugs together.

It is important to realize that the CODE-2 Study only captures part of the cost of the diabetes, as only direct healthcare costs are included. Lost production, caused by sick absence, early retirement, and early mortality also carries high costs. In studies that have included this component, it sums up to more than 50% of the total costs.[696,697]

The cost-effectiveness of intervention

Knowledge about the cost of a disease is not sufficient information to guide us when making a decision on how to treat it. To do that, we need information about the cost of the intervention, and about the expected health effects. In other words, we need information about the cost-effectiveness of the interventions. There have been many studies investigating the cost-effectiveness of different treatment strategies for diabetic patients. Here we will focus on the prevention of macrovascular complications, as they are the largest contributor to the costs associated with the disease.

Lipid-lowering using statins in diabetics have been studied in several studies. In a subgroup of the 4S Trial, cost-effectiveness ratios of treating diabetic patients with 20–40 mg simvastatin were found to be well below the levels that are usually considered cost-effective.[708] Diabetic patients were also enrolled in the HPS, which indicated acceptable cost-effectiveness ratios for patients with this risk level.[709] One important thing to consider about these studies is that they used a cost of simvastatin prior to the expiry date of the patent. Thereafter the price dropped substantially which would mean that statin use in diabetics is likely to be cost-

Table 27 Distribution of annual per-patient costs by main resource category in Euro[705]

Country	Resource category			
	Hospitalization (mean ± SD)	Ambulatory (mean ± SD)	Oral anti-diabetic drugs only (mean ± SD)	All other drugs (mean)[a]
Belgium	1791 ± 5864	603 ± 931	127 ± 114	774
France	1540 ± 6252	683 ± 1433	207 ± 169	633
Germany	2173 ± 755	388 ± 47	119 ± 8	896
Italy	1787 ± 8778	555 ± 516	63 ± 71	586
The Netherlands	548 ± 3570	450 ± 1307	102 ± 118	734
Spain	417 ± 1960	334 ± 307	61 ± 101	494
Sweden	1116 ± 6135	813 ± 1088	41 ± 37	661
United Kingdom	769 ± 4015	835 ± 775	60 ± 71	519
CODE-2 Average	1333	603	103	476

[a]No SD values are available because of the method of calculation.

saving in secondary prevention and associated with very low cost-effectiveness ratios in primary prevention.

Another approach to prevention of macrovascular complication is through blood pressure control. This has been studied as part of the UKPDS, where tight blood pressure control using BBs and ACE-inhibitors was investigated. A recent cost-effectiveness analysis of this intervention indicated that this treatment strategy was associated with a very high cost-effectiveness.[710] In another study, Casciano *et al.* investigated the cost-effectiveness of doxazosin in Italy and the UK, and also found acceptable cost-effectiveness ratios.[711]

It can be concluded that the costs associated with diabetes make up a considerable share of the resources spent on healthcare throughout Europe. As the most important cost drivers are complications caused by the disease, proper management in the prevention of complications is essential.

Recommendation

Lipid-lowering treatment provides a cost-effective way of preventing complications. Class I, Level of Evidence A.

Tight control of hypertension is cost-effective. Class I, Level of Evidence A.

Appendix

Glossary of abbreviations and acronyms

AACE, American Association of Clinical Endocrinologists; **ACC**, American College of Cardiology; **ACCP**, American College of Chest Physicians; **ACS**, Acute Coronary Syndromes; **ADA**, American Diabetes Association; **ADP**, Adenosine Diphosphate; **AGEs**, Advanced Glycation End Products; **AHA**, American Heart Association; **ALFA**, Activité Libérale sur la Fibrillation Auriculaire; **AMI**, acute myocardial infarction; **ARB**, angiotensin receptor blocker; **ATP**, adenosine triphosphate; **BB**, beta-blocker; **BMI**, body mass index; **CABG**, coronary artery bypass grafting; **CAD**, coronary artery disease; **CAS**, carotid artery angioplasty and stenting; **CCB**, calcium channel blocker; **CI**, confidence interval; **COX-2**, cyclooxygenase-2; **CPG**, Committee for Practice Guidelines; **CS-D**, cardiac surgery in diabetes patients; **CV**, cardiovascular; **CVD**, cardiovascular disease(s); **DAG**, diacylglycerol; **DBP**, diastolic blood pressure; **DES**, drug eluting stents; **DM**, diabetes mellitus; **DMF**, diabetes determined by fasting plasma glucose ≥7.0 mmol/L and 2-h plasma glucose <11.1 mmol/L; **DMF and DMP**, diabetes determined by fasting plasma glucose ≥7.0 mmol/L and 2-h plasma glucose ≥11.1 mmol/L; **DMP**, diabetes determined by 2-h plasma glucose ≥11.1 mmol/L and fasting plasma glucose <7.0 mmol/L; **EASD**, European Association for the Study of Diabetes; **ECG**, electrocardiogram; **EGIR**, European Group for the Study of Insulin Resistance; **eNOS**, endothelial nitric oxide synthase; **ESC**, European Society of Cardiology; **F**, female; **FFA**, free fatty acid; **FPG**, fasting plasma glucose; **GAD**, glutamic-acid-decarboxylase; **GIK**, glucose–insulin–potassium; **GLUT**, glucose transporters; **HbA_{1c}**, glycated haemoglobin; **HDL**, high-density lipoprotein; **HEED**, Health Economics Evaluation Database; **HF**, heart failure; **HOT**, hypertension optimal treatment; **HR**, hazard ratio; **ICU**, intensive care unit; **IDF**, International Diabetes Federation; **IFG**, impaired fasting glucose/glycaemia; **IGR**, impaired glucose regulation; **IGT**, impaired glucose tolerance; **ILs**, interleukins; **INR**, International normalized ratio; **LDL**, low-density lipoprotein; **M**, male; **MAPK**, mitogen-activated protein kinase pathway; **MCP-1**, monocyte chemoattractant; **MI**, myocardial infarction; **MODY**, maturity-onset diabetes in the young; **NADPH Ox**, nicotinamide adenine dinucleotide phosphate oxidase; **NCEP**, National Cholesterol Education Programme; **NIDDK**, National Institutes of Diabetes, Digestive, and Kidney Diseases; **NNT**, numbers needed to treat; **NO**, nitric oxide; **NOS**, nitric oxide synthase; **NSTEMI**, non-ST-elevation myocardial infarction; **OGTT**, oral glucose tolerance test; **ONOO**, peroxynitrite; **PAI**, plasminogen activator inhibitor; **PCI**, percutaneous coronary interventions; **PGI_2/TXB_2**, prostacyclin/thromboxane; **PGIS**, prostacyclin synthase; **PI-3K**, phosphatidylinositol-3 kinase; **PKC**, protein kinase; **PLC**, phospholipase; **RAS**, renin–angiotensin system; **RITA**, randomized intervention treatment of angina; **ROS**, reactive oxygen species; **RR**, risk ratio; **SCD**, sudden cardiac death; **SD**, standard deviation; **SK**, streptokinase; **SPECT**, single-photon emission computed tomography; **STEMI**, ST-elevation myocardial infarction; **TC**, total cholesterol; **TCD**, transcranial Doppler; **TG**, triglyceride; **Thr**, thrombin; **TIA**, transient ischaemic attacks; **TNF**, tumour necrosis factor; **tPA**, tissue plasminogen activator; **Tz**, thiazide; **TZD**, thiazolidinediones; **UA**, unstable angina pectoris; **VLDL**, very low-density lipoprotein; **WHO**, World Health Organization; **DECODE Study**, Diabetes Epidemiology: Collaborative Analysis of Diagnostic Criteria in Europe; **4S**, Scandinavian Simvastatin Survival Study; **ABCD**, Appropriate Blood Pressure Control in Diabetes; **ACCORD**, Angioplastie Coronaire, Corvasal and Diltiazem; **ACE-I**, angiotensin converting enzyme-inhibitor; **ADDITION study**, Anglo-Danish-Dutch Study of Intensive Treatment and Complication Prevention in Type 2 Diabetic Patients Identified by Screening in Primary Care; **ALLHAT**, Anti-hypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial; **ARIC**, Atherosclerotic Risk in Community Study; **ARTS**, Arterial Revascularization Therapy Study; **ASCOT**, Anglo-Scandinavian Cardiac Outcomes Trial; **ASCOT-LLA**, Anglo-Scandinavian Cardiac Outcomes Trial–Lipid Lowering Arm; **ATLAS**, Assessment of Treatment with Lisopronil and Survival; **ATP III**,

Adult Treatment Panel III; **AWESOME**, Angina with Extreme Serious Operative Mortality Evaluation; **BARI**, Bypass Angioplasty Revascularization Investigation; **CABRI**, Coronary Angioplasty vs. Bypass Revascularization Investigation; **CAPPP**, Captopril Prevention Project; **CAPRIE**, Clopidogrel vs. Aspirin in Patients at Risk of Ischaemic Events; **CARDia**, Coronary Artery Revascularization in Diabetes Trial; **CARDS**, Collaborative Atorvastatin Diabetes Study; **CARE**, Cholesterol and Recurrent Events Substudy; **CHADS**, Congestive heart failure, Hypertension, Age >75 years, Diabetes, prior Stroke or TIA; **CHARISMA-Study**, Clopidogrel for high atherothrombotic risk and ischaemic stabilization management and avoidance; **CIBIS II**, The Cardiac Insufficiency Bisoprolol Study II; **CODE 2 Study**, Cost of type II Diabetes in Europe; **COMET**, Carvedilol Or Metopropol European Trial; **CONSENSUS**, Cooperative North Scandinavian Enalapril Survival Study; COPERNICUS, Carvedilol Prospective Randomized Cumulative Survival; **CURE**, Clopidogrel in Unstable Angina To Prevent Recurrent Events; **DCCT**, Diabetes Control and Complication Trial; **DIGAMI**, Diabetes Glucose And Myocardial Infarction; **EAST**, Emory Angioplasty vs. Surgery Trial; **ECLA**, Estudios Cardiologicos Latinoamerica; **EDIC Study**, Epidemiology of Diabetes Interventions and Complications; **EHS-ACS**, The Euro Heart Survey of Acute Coronary Syndromes; **EUROASPIRE I, II**, European Action on Secondary Prevention through Intervention to Reduce Events I, II; **EURODIAB IDDM Complication Study**, EURODIAB Insulin Dependent Diabetes Mellitus Complication Study; **EUROPA**, EUropean Trial on Reduction Of cardiac events with Perindopril in stable coronary Artery disease; **FACET**, Fosinopril vs. Amlodipine Cardiovascular Events Randomized Trial; **FIELD**, Fenofibrate Intervention and Event Lowering in Diabetes; **FINDRISC**, FINnish Diabetes Risk Score; **FREEDOM**, Future Revascularization Evaluation in Patients with Diabetes Mellitus: Optimal Management of Multivessel Disease; **FRISC-II**, Fragmin and Fast Revascularization During Instability in Coronary Artery Disease II; **GISSI**, Gruppo Italiano per lo Studio della Sopravvivenza nell' Infarto miocardio; **GISSI 3**, The Third Gruppo Italiano per lo Studio della Sopravvivenza nell' Infarto miocardio; **GRACE**, Global Registry of Acute Coronary Events; **GUSTO-I**, Global Utilization of Streptokinase and tPA for Occluded Coronary Arteries-I; **GUSTO-IIb**, Global Utilization of Streptokinase and tPA for Occluded Coronary Arteries-IIb; **HDFP**, Hypertension Detection and Follow-up Program; **HHS**, Helsinki Heart Study; **HOPE**, Heart Outcomes Prevention Evaluation; **HPS**, Heart Protection Study; **INSIGHT**, International Nifedipine GITS Study Intervention as a Goal in Hypertension Treatment; **LIFE**, Losartan Intervention for Endpoint reduction in hypertension Study; **MATCH-Trial**, Antiplatelets in Stroke Prevention; **MERIT-HF**, Metopropol Randomized Intervention Trial in Congestive Heart Failure; **MITRA**, Maximal Individual Therapy in Acute myocardial infarction; **MONICA**, Monitoring trends and determinants in Cardiovascular disease; **NHANES II**, National Health and Nutrition Examination Survey; **NHANES III Study**, The Third National Health and Nutrition Examination Survey; **NORDIL**, The Nordic Diltiazem; **OASIS**, Organization to Assess Strategies for Ischemic Syndromes; **PROACTIVE Trial**, a randomized controlled trial of the efficacy of a family-based, domiciliary intervention programme to increase physical activity among individuals at high-risk of diabetes; **PROCAM**, Prospective Cardiovascular Munster; **PROGRESS**, Perindopril Protection Against Recurrent Stroke Study; **PROVE-IT Trial**, Pravastatin or Atorvastatin Evaluation and Infection Therapy; **RCT**, Randomized Controlled Trial; **RIKS-HIA**, Register of Information and Knowledge about Swedish Heart Intensive Care Admission; **SAVE**, Survival And Ventricular Enlargement Study; **SHEP**, Systolic Hypertension in the Elderly Program; **SOLVD**, Studies of Left Ventricular Dysfunction; **SPATRIAL**, Stroke Prevention in Atrial fibrillation III Study; **STENO 2**, Cardiovascular Disease and Type 2 Diabetes; **STOP-2**, Swedish Trial in Old Patients with Hypertension-2; **STOP-NIDDM Trial**, Study TO Prevent Non-Insulin-Dependent Diabetes Mellitus; **Syst-EUR**, Systolic Hypertension in Europe Trial Investigators; **TNT**, Treat to New Targets Trial; **UKPDS**, United Kingdom Prospective Diabetes Study; **VAHIT**, Veterans Administration HDL Trial; **VALIANT**, VALsartan In Acute myocardial iNfarcTion.

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APPENDIX B

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Characterization of Hydroxylamine-Cytochrome *c* Reductase from the Chemoautotrophs *Nitrosomonas europaea* and *Nitrosocystis oceanus**

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Until recently studies on the mechanism of nitrification by the chemoautotrophic bacterium *Nitrosomonas* have been limited for the most part by the difficulty in obtaining large numbers of cells in pure culture. Lees (1), using a growth medium containing a precipitate of the phosphates and carbonates of calcium, magnesium, and iron, showed that several chelators (including salicylaldoxime, sodium diethyldithiocarbamate, and allylthiourea) inhibited the production of nitrite from ammonia by whole cells. Later, Hofman and Lees (2) implicated hydroxylamine as an intermediate in the nitrification process by demonstrating *in vivo* that hydroxylamine was converted to nitrite and that, in the presence of hydrazine which inhibited this conversion, hydroxylamine accumulated from ammonia. The subsequent development by Engel and Alexander (3) of an entirely soluble growth medium giving appreciable yields of *Nitrosomonas* has made possible more extensive investigations of this organism.

Nicholas and Jones (4) reported that cell-free extracts prepared by sonic oscillation of *Nitrosomonas* catalyzed the hydrazine-inhibited conversion of hydroxylamine to nitrite in the presence of such electron acceptors as mammalian cytochrome *c* or phenazine methosulfate. Hydrazine in the presence of extracts also reduced cytochrome *c*. Falcone, Shug, and Nicholas (5) later observed that a particulate *Nitrosomonas* fraction sedimenting after 10 hours at 100,000 × *g* catalyzed nitrite formation from hydroxylamine in the presence of oxygen and catalytic amounts of cytochrome *c*. Under anaerobic conditions 2 moles of cytochrome *c* were reduced per mole of hydroxylamine added, and nitrous oxide, rather than nitrite, was evolved. The presence in the particulate preparation of cytochromes of the *b*, *c*, and *a* type as well as a flavin was also indicated. The fact that atebrin inhibited cytochrome *c* reduction by NH_2OH and that this effect was prevented by flavin adenine dinucleotide implicated a flavin involvement. Anderson (6) reported the anaerobic conversion of hydroxylamine to NO and N_2O by *Nitrosomonas* extracts in the presence of methylene blue. In addition, the extracts catalyzed the uptake of NO in the presence of ferricyanide. Burge, Malavolta, and Delwiche (7) observed that hydroxylamine stimulates ^{32}P-labeled phosphate incorporation into adenosine triphosphate as catalyzed by the 100,000 × *g* supernatant solution of *Nitrosomonas* extracts prepared by sonic oscillation.

The present work compares the properties of the electron transport systems involved in hydroxylamine oxidation by the chemautotrophs *Nitrosomonas europaea* and *Nitrosocystis oceanus*. It includes the partial purification and characterization of the hydroxylamine-cytochrome *c* reductase system from each organism. The corresponding systems from the two organisms appear to be similar in most of the properties examined. A preliminary summary of this work has already been reported (8).

EXPERIMENTAL PROCEDURE

Growth of Bacteria—Nitrosomonas europaea (originally obtained from Dr. B. Pramer of Rutgers University) was routinely grown at 25° in 15 1-liter volumes of nutrient medium according to a modification of the procedure of Engel and Alexander (3). A volume of 15 liters of growth medium, pH 8.0, containing, per liter, 13.4 g of Na_2HPO_4, 0.77 g of KH_2PO_4, 0.5 g of $NaHCO_3$, and 2.5 g of $(NH_4)_2SO_4$ was made up in a 20-liter glass or Nalgene carboy and autoclaved at 121° for 1 hour. A 30-ml volume of a separately autoclaved solution of $Mg_2SO_4 \cdot 7H_2O$ (250 g per liter), $CaCl_2 \cdot 2H_2O$ (9.2 g per liter), and Chel-138 HFe, (Geigy Chemical) (50 mg per ml) was added to the 15-liter solution immediately before it was inoculated with a 1-liter culture of bacteria. To prepare the 1-liter inoculum, a 100-ml volume of complete growth medium was first inoculated with 0.1 to 0.5 ml of solution from another 100-ml culture which was already in the log phase of growth. When the recipient 100-ml culture attained the log phase of growth (in approximately 3 days), it was transferred into 1 liter of growth medium in a 2.3-liter Fernbach flask and again incubated until the log phase of growth was reached. Cultures were vigorously aerated by shaking at 60 rpm on a reciprocating shaker (New Brunswick Scientific) in the case of the Erlenmeyer and Fernback flasks, and in the carboys by forced aeration through sintered glass tips. The pH of the 15-liter batch cultures was maintained between 7.5 and 8 during growth by the addition of 50% K_2CO_3 which had been sterilized by passage through a millipore filter. Growth was followed by nitrite production as assayed colorimetrically in successive 0.1-ml aliquots taken from the culture medium during the course of incubation.

The cells were usually harvested 6 days after inoculation when the amount of nitrite nitrogen produced (final nitrite concentra-

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tion, 0.02 M) was equivalent to half of the original ammonia nitrogen in the growth medium. Cells from 90 liters of growth medium were then sedimented in a Sharples refrigerated continuous flow centrifuge with a flow rate of 20 to 30 liters per hour and washed by suspension in 500 ml of 0.1 M Tris buffer, pH 8.0 Sigma) and centrifugation at 10,000 × *g* for 15 min at 4°.[1] The washing procedure was repeated to yield a characteristic brownish red pellet of approximately 12 g (fresh weight), which was stored at −4°.

Batch cultures of *Nitrosocystis oceanus* sp. n. (9) were grown and harvested by Dr. S. W. Watson at the Woods Hole Oceanographic Institution in a salt medium similar to that used for *Nitrosomonas* except that sea water made sterile by millipore filtration was employed in place of distilled water. An automatic continuous titration apparatus was employed to maintain the pH at 7.5. Cells were harvested by continuous flow centrifugation (Servall); washed twice with 3% NaCl in 0.05 M phosphate, pH 7.5; collected by centrifugation for 15 min at 10,000 × *g*; suspended in a minimum of 0.01 M phosphate, pH 7.5; freeze-dried; and stored at −4°. The average yield of approximately 55 g, fresh weight, per 90 liters of culture medium was several fold greater than that obtained for *Nitrosomonas*. The contamination, which occurred with *Nitrosocystis* cultures of high cell density, was estimated by Dr. Watson to be less than 1%. Engel and Alexander (3) found by plating cultures on nutrient broth that heterotrophic contamination of less than 1% occurred in *Nitrosomonas europaea* grown essentially as described here.

Substrates, Cofactors, and Other Substances—Separate solutions of hydroxylamine hydrochloride (Baker "analyzed" reagent) and hydrazine hydrochloride (Fisher certified) were prepared daily by dilution from 0.1 M stock solutions, which were kept at 0–4° for no longer than 3 weeks and which showed no decomposition or trace of nitrite. Ammonium sulfate and sodium or potassium nitrite (Baker analyzed reagent) solutions were prepared weekly and stored at 4°. Aqueous solutions of DCI[2] (sodium salt, Eastman) and potassium ferricyanide (Baker analyzed reagent) were prepared daily. All substrates were employed without neutralization except when used at levels high enough to alter the final pH of the reaction mixture.

Unless otherwise noted, most of the studies employed aqueous solutions of type III horse heart cytochrome *c* (95 to 100% pure, from Sigma) which were prepared weekly and stored at 4°. Type II horse heart cytochrome *c* (60 to 70% pure, Sigma) was used in assays during purification of the enzymes. Type II cytochrome *c* was reduced by the hydrogen-palladium asbestos procedure as described by Smith (10). NADH (98 to 100% pure), NADPH (98% pure), NADP (94% pure), FAD (90 to 94% pure), FMN (100% pure), and pyridoxal phosphate (99% pure) were obtained from Sigma; NAD from Pabst; and crystalline bovine serum albumin from the Mann Research Laboratories. Antimycin A and sodium Amytal were supplied by the Wisconsin Alumni Research Foundation and Eli Lilly, respectively; the compound, 4-hydroxy-2-*n*-heptyl quinoline *N*-oxide, was kindly provided by Dr. Lester Packer. Iodoacetic acid, sodium arsenate and arsenite, and 2,4-dinitrophenol were obtained from Distillation Products, Ltd., Merck, and Mathieson, Coleman and Bell, respectively; atebrin was a gift of Dr. Leslie Hellerman. Calcium phosphate gel was prepared according to the procedure of Keilin and Hartree (11) except that distilled water was used. Sephadex G-200 was purchased from Pharmacia, and hydroxylapatite from the Clarkson Chemical Company as Hypatite-C. Whatman DE-50 DEAE-cellulose was washed and prepared for use in 40-g batches by successive suspension as follows: (*a*) several times in water followed by decantation of the "fines," (*b*) twice in 1 liter of 0.25 N NaOH followed by water, (*c*) twice in 500 ml of 0.1 N HCl followed by water, (*d*) twice in 0.25 N NaOH, (*e*) in water until the pH approached 7.0, and (*f*) in the appropriate buffer until properly equilibrated.

[1] During the first wash a white precipitate denser than the cells was sometimes observed from which the cells could be separated easily with a rubber policeman.

[2] The abbreviations used are: DCI, 2,6-dichlorophenolindophenol; BSA, bovine serum albumin; HMB, *p*-hydroxymercuribenzoate.

Assay Procedures

Hydroxylamine, Nitrite, and Protein—The hydroxylamine content of 1 to 2 ml of solution contained in a test tube (15 × 125 mm) was assayed by the method of Frear and Burrell (12). Nitrite did not affect color formation in this assay. Ferric chloride and horse heart cytochrome *c* caused increases of absorbance but did not interfere with evaluation of *differences* in hydroxylamine levels. Although the standard curve of hydroxylamine concentration with respect to absorbance was always linear, the intensity of color formation per mole of hydroxylamine varied over a 2-fold range which required that a standard curve be run with each experiment.

Nitrite was assayed colorimetrically by the diazo-coupling procedure essentially as described by Nicholas and Nason (13). Neither DCI, whether oxidized or reduced, nor allylthiourea had an effect on color formation in the assay. Reduced and oxidized cytochrome *c* absorbed equally under the conditions of the assay but apparently did not interfere with the evaluation of *changes* in nitrite levels.

Samples for protein assay during enzyme purification were prepared by precipitating approximately 200 μg of protein at 0° in 1 ml of 7% trichloroacetic acid. The resulting pellet was resuspended in 2 ml of 2% Na_2CO_3 in 0.1 N NaOH, and suitable aliquots were assayed for protein by the Lowry method as described by Layne (14) with crystalline BSA as the standard. For any particular purification step the absorbance at 280 mμ was considered a satisfactory estimation of relative protein concentration in the collected fractions provided the ratio of absorbance at 280 mμ to absorbance at 260 mμ was the same and greater than 1.0 in all such fractions.

Difference spectra were measured in a Cary model 14 recording spectrophotometer with the use of the sensitive slide wire (0 to 0.2 absorbance unit) and a 1-cm light path.

Standard Enzyme Assays—All enzyme assays were conducted at room temperature. The rates of enzymatic reduction by hydroxylamine or hydrazine of cytochrome *c*, DCI, and ferricyanide, and oxidation of reduced mammalian cytochrome *c* by molecular oxygen were measured in a Beckman DU spectrophotometer with a 1-cm light path. Absorbance readings were typically taken 30 sec after the start of the reaction and at 30-sec intervals thereafter for 3 min with a level of enzyme giving an absorbance change within the range of 0.05 to 0.20 unit/2 min. The rate of a control reaction mixture from which enzyme had been omitted was measured simultaneously and subtracted from that in the presence of enzyme to correct for any nonenzymatic reaction. The rate of an additional control reaction

mixture from which hydroxylamine or hydrazine was excluded was also used, where required, to correct for endogenous reduction of electron acceptor. The rate of change in absorbance was linear during the time observed and proportional to the amount of enzyme added. Anaerobic experiments were conducted under N_2 gas (purified by bubbling twice through a solution of 2 mM methylene blue containing an excess of powdered metallic zinc) in Thunberg cuvettes which had first been evacuated to approximately 5 mm of Hg for at least 3 min. The reaction was started by tipping enzyme in from the side arm.

The cytochrome *c* reductase reaction was started by the addition of 0.05 ml of a 2 mM solution of either NH_2OH or NH_2NH_2 to a cuvette containing 0.05 ml of 1.5 mM type III horse heart cytochrome *c*, 0.01 to 0.2 ml of enzyme solution, and enough 0.05 M glycine-NaOH buffer, pH 9.6, to give a total volume of 1.0 ml. The resulting rate of increase in absorbance at 550 mμ was measured as described above. Under these conditions there was little or no endogenous or nonenzymatic reduction of cytochrome *c*. Appreciable nonenzymatic reduction did occur, however, at progressively higher concentrations of substrate and cytochrome *c*. Changes in concentration of cytochrome *c* expressed on a molarity basis were calculated from an extinction coefficient (reduced minus oxidized) of 2.10×10^4 M^{-1} cm^{-1} (15) which was found not to vary over the pH range studied. One unit of NH_2OH-cytochrome *c* reductase activity is defined as that amount of enzyme which catalyzes the reduction of 1.0 μmole of mammalian cytochrome *c* per min at room temperature. Specific activity is expressed as units of enzyme activity per mg of protein.

The concomitant disappearance of hydroxylamine and reduction of cytochrome *c* was followed in a 10-ml reaction mixture (contained within a larger cuvette) with the same concentrations of constituents as described above except that the initial concentration of hydroxylamine ranged from 20 to 200 μM depending upon the experiment. The rate of hydroxylamine disappearance was measured in 0.5- to 2-ml aliquots pipetted from the above reaction mixture into test tubes (15 × 125 mm) containing 0.2 ml of 12% TCA at regular intervals during the course of the reaction. The rate of disappearance of hydroxylamine in the absence of enzyme was measured and subtracted from the rate with enzyme.

The assay of DCI-reductase was essentially the same as that for cytochrome *c* reductase described above except that 0.01 M potassium phosphate buffer, pH 7.0; 0.05 ml of 2×10^{-3} M DCI; and 0.05 ml of 10^{-2} M NH_2OH were used in place of glycine buffer, cytochrome *c*, and 0.05 ml of 2×10^{-3} M NH_2OH, respectively. The resulting rate of decrease in absorbance was measured at 600 mμ.

The potassium ferricyanide reductase assay was also essentially the same as that for cytochrome *c* reductase except that glycine buffer, pH 8.5, and 0.05 ml of 0.02 M potassium ferricyanide were used in place of buffer, pH 9.6, and cytochrome *c*, respectively. The resulting rate of decrease in absorbance was measured at 420 mμ.

The cytochrome *c* oxidase reaction was started by the addition of 0.01 to 0.2 ml of enzyme solution to a cuvette containing 0.02 ml of 2.5×10^{-3} M type II reduced horse heart cytochrome *c* and 0.05 M glycine-NaOH (pH 9.6) or potassium phosphate (pH 7.5 or 6.8) to give a total volume of 1.0 ml. The resulting rate of decrease in absorbance was measured at 550 mμ.

TABLE I

Summary of purification of NH_2OH-cytochrome c reductase from Nitrosomonas europaea

Fraction	Activity	Protein	Specific activity	Recovery during purification step	$A_{420}:A_{280}$
	units/ml	*mg/ml*	*units/mg protein*	%	
1. Crude extract	7.6	3.64	2.1	100	0.70
2. Calcium phosphate gel eluate	2.1	0.38	5.5	82	0.84
3. DEAE-cellulose eluate	13.4	1.15	11.6	70*	1.08
4. $(NH_4)_2SO_4$ fraction	154	6.5	23.7	83	1.10
5. Sephadex G-200 eluate	10.6			79	1.09
6. Hydroxylapatite eluate	0.74	0.026	28.5	68	0.94

* A large amount of enzyme activity was accidentally lost during the particular DEAE-cellulose treatment described in the table. This value of percentage recovery is based on a typical recovery of 70% in the DEAE-cellulose step.

RESULTS

Purification of Hydroxylamine-Cytochrome c Reductase—Unless otherwise indicated, all steps of the purification process were carried out at 4°.

From Nitrosomonas—The pertinent data of a typical purification of the *Nitrosomonas* enzyme described in detail below are summarized in Table I. Fresh cells or cells stored at −10° were suspended in 0.1 M Tris buffer, pH 8.0, in a ratio of 5 g of cells to 100 ml of buffer, by gentle mixing in a loosely fitting TenBroeck glass homogenizer and disrupted by sonic oscillation in 40-ml batches for 15 min in a Raytheon sonicator with the use of maximum power. The resultant extract showed a characteristic increased redness and decreased viscosity. It was centrifuged for 15 min at 10,000 × *g* to yield a pellet (containing unbroken cells, cell debris, and sometimes a pasty white precipitate) which was discarded. The supernatant solution was centrifuged at 100,000 × *g* for 2 hours in a Spinco model L centrifuge to give a red-brown, tightly packed pellet and a clear reddish supernatant solution. The latter, which usually contained 70% or more of the hydroxylamine-cytochrome *c* reductase activity, was designated as the crude cell-free enzyme (Fraction 1).

Fraction 1 was dialyzed three times, each time for 5 hours against 9 volumes of 0.1 M Tris, pH 8.0. To 850 ml of dialyzed Fraction 1 were added 192 ml of calcium phosphate gel suspension (40 mg, dry weight, per ml) in 5- to 10-ml portions with continuous stirring. The mixture was allowed to stand with continuous gentle mixing for 60 min before centrifugation at 1500 × *g* for 20 min. The resultant gel-pellet which contained 90% of the enzyme activity was washed three times by dispersing the gel in 850-ml volumes of 0.07 M potassium phosphate, pH 8.0, allowing it to stand for 60 min with continuous stirring, and centrifuging. The enzyme was then eluted from the gel by the same procedure with the use of 3 successive 870-ml volumes of 0.38 M potassium phosphate, pH 8.0. The combined 0.38 M phosphate eluates (Fraction 2) displayed a 3- to 10-fold increase in specific activity with recoveries of 50 to 90% of the activity of Fraction 1 depending on the age and source of the gel and enzyme

preparation. It was found necessary on the basis of preliminary trials with 1- to 5-ml portions of Fraction 1 to modify the ratio of gel to protein and the number and buffer molarity of elutions as different batches of gel and crude enzyme were used.

The potassium phosphate concentration of Fraction 2 was changed from 0.38 M to an estimated 1.0 mM by successive dialysis against distilled water over periods of 10 to 12 hours. Dialyzed Fraction 2 was then added at the rate of 3 drops per min to a chromatography column (4 × 40 cm) which had been packed under pressure with washed DEAE-cellulose and previously equilibrated with 1.0 mM potassium phosphate, pH 7.5. A red-brown area which apparently corresponded to the enzyme activity remained at the upper half of the column during subsequent perfusion with 1 liter of 1.0 mM potassium phosphate at pH 7.5. A linear gradient elution procedure was then employed at the rate of 3 drops per min between 2 liters of 0.01 M phosphate, pH 7.5, and 2 liters of 0.5 M KCl in 10^{-3} M phosphate at pH 7.5. In this manner enzyme activity was eluted in a slightly asymmetric peak beginning at approximately 0.2 M KCl. The DEAE-cellulose eluate fractions were pooled to form a red solution (Fraction 3), containing 70% of the Fraction 2 enzyme units with a specific activity 1.5 to 2.5 times that of Fraction 2.

Fraction 3 was dialyzed twice for 4 hours, each time against 100 volumes of 0.01 M potassium phosphate, pH 7.5. Successive amounts of solid ammonium sulfate were added slowly to Fraction 3 to give the following concentrations in grams per ml: 0.15, 0.2, 0.3, 0.4, 0.5, and 0.6. Each precipitate was collected by centrifugation after standing for 1 hour at 0°. The red precipitates (collected between 0.3 and 0.5 g of $(NH_4)_2SO_4$ per ml) containing 80 to 90% of the activity of Fraction 3 were resuspended in a minimum of 0.01 M potassium phosphate, pH 7.5, and centrifuged to yield a clear supernatant solution (Fraction 4).

For the next purification step a column of Sephadex G-200 (1 × 20 cm) was prepared as follows. A suspension of 2 g of Sephadex G-200 dispersed in 250 ml of 0.1 M KCl-0.01 M potassium phosphate solution, pH 7.5, which had been decanted free of fine particles and equilibrated for 2 to 3 days, was allowed to settle in the column until a 2-cm layer had formed at the bottom. The phosphate-KCl-Sephadex suspension was then allowed to flow through the column at a rate of 5 drops per min. When the column had been fully packed and had equilibrated for 6 hours, a relatively firm 5-mm layer of Sephadex G-25 suspended in the same buffer was deposited at the top of the Sephadex G-200 in order to facilitate the subsequent addition of enzyme without disturbing the packing of Sephadex G-200. The column was equilibrated by allowing phosphate-KCl solution to flow through overnight. Solid KCl and sucrose were dissolved in Fraction 4 to a final concentration of 0.1 M and 25%, respectively, and 0.4 ml of this solution was layered on top of the Sephadex G-25 through the overlying 2 to 3 cm of phosphate-KCl solution. As the phosphate-KCl solution flowed through the column at 3 drops per min, the enzymatic activity moved in a single red band. The enzyme was eluted after the passage of a volume of effluent approximately equivalent to the void volume of the column to yield Fraction 5, with total and specific activities of 80 and 114%, respectively, of Fraction 4.

Fraction 5 was dialyzed for 10 hours against 500 volumes of 2 mM potassium phosphate buffer, pH 7.5, added at the rate of 2 drops per min to a gravity-packed Hypatite column (1 × 20 cm) which had been previously equilibrated with 2 mM potassium phosphate, pH 7.0. A linear gradient elution was used between 250-ml volumes of 2 mM and 1.0 M potassium phosphate buffer, pH 7.5, resulting in the removal of the enzyme (Fraction 6) in a symmetrical peak at around 0.3 M potassium phosphate, with total and specific activities of 68 and 106%, respectively, of Fraction 5. *Nitrosomonas* Fraction 6 had a turnover number of 2,850 moles of cytochrome *c* reduced per min per mole of enzyme, on the assumption of a molecular weight of 100,000 for the NH_2OH-cytochrome *c* reductase.

TABLE II

Summary of purification of NH_2OH-cytochrome c reductase from Nitrosocystis oceanus

Fraction	Protein	Specific activity	Total activity	A_{280}: A_{420}
	mg/ml	*units/mg protein*	*units*	
1. Crude extract	12	0.43	310	
2. 70° heat-treated $(NH_4)_2SO_4$ fraction	1.5	2.0	167	0.42
3. pH precipitate	5.0	3.6	162	0.60
4. $(NH_4)_2SO_4$ precipitate	13	9.5	186	0.70
5. Sephadex G-200 eluate	0.95	9.0	86	1.06
6. DEAE-cellulose eluate	0.71	10	76	
7. Hydroxylapatite eluate	0.14	13	52	1.10

From Nitrosocystis—The pertinent data of the purification of the *Nitrosocystis* hydroxylamine-cytochrome *c* reductase described in detail below are summarized in Table II.

To prepare a crude extract, 70 to 90 g of washed *Nitrosocystis*, or its equivalent as lyophilized cells, were first suspended overnight in 100 ml of 0.01 M potassium phosphate solution, pH 7.5. The suspension was then disrupted by sonic oscillation for 5 min in 30-ml batches in an ice-cooled Raytheon sonicator, or in 15-ml batches with the Branson sonifier at full power with simultaneous cooling by submersion in an ice-ethanol-NaCl mixture. The pellet resulting from centrifugation of the disrupted suspension at 20,000 × *g* for 20 min was either discarded or treated again with the Branson sonifier in a minimal volume of the phosphate buffer (15 to 20 ml), and the combined supernatant solutions were centrifuged at 100,000 × *g* for 2 hours. The resultant pellet was discarded, and the supernatant solution was designated as the crude extract (Fraction 1).

Powdered ammonium sulfate was added slowly with stirring to Fraction 1 in a ratio of 4 g per ml. The pH of the suspension was then adjusted to 6.3 with 1 N NaOH, and the temperature was brought to 70° and maintained for 14 min in a water bath. After being cooled rapidly to 0° in an ice-ethanol-NaCl mixture, the preparation was kept at 0° for 60 min and centrifuged at 5000 × *g* for 30 min. The resulting precipitate was extracted with 32% ammonium sulfate in 0.01 M potassium phosphate solution (pH 6.3) equal to half of the starting volume of Fraction 1, and the original supernatant solution and extract were combined to yield Fraction 2. The latter contained 55 to 91% of the activity and was 5- to 7-fold purified (as compared to the crude extract). Fraction 2 was adjusted to pH 5.0 with 2 N acetic acid and allowed to stand for 15 hours, and the resultant precipitate was collected by centrifugation and suspended in a minimum volume of 1 mM potassium phosphate solution, pH 7.5, to give Fraction 3. The latter contained 54% of the enzyme units with an 8-fold purification as compared to Fraction 1.

Ammonium sulfate was added to Fraction 3 in the ratio of 0.58 g per ml, and the mixture was allowed to stand at 0–4° for 1 hour with intermittent stirring. The resultant heavy red precipitate was sedimented by centrifugation and resuspended in about 3 ml of 0.01 M potassium phosphate-0.01 M KCl solution (pH 7.5) to constitute Fraction 4, which possessed 60% of the total enzyme units with a 22-fold purification as compared to Fraction 1.

Fraction 4 was passed through a Sephadex G-200 column (2.4 × 24 cm) essentially as described for the *Nitrosomonas* enzyme to yield three elution peaks of 280 mμ absorbance. The small first peak and the large third peak had little or no activity and an $A_{280}:A_{260}$ ratio of 0.6, indicating a high nucleic acid content. The second and largest peak, which followed closely after the first, contained the activity and had an $A_{280}:A_{260}$ ratio of slightly more than 1.0. The pooled eluates of the active peak (Fraction 5) had 28% of the enzyme units, and Fraction 5 was 21-fold purified as compared to Fraction 1.

Fraction 5 was dialyzed for 6 hours against three changes of 100 volumes of 1 mM potassium phosphate solution (pH 7.5) and added to a DEAE-cellulose column (1.1 × 13 cm) previously packed under pressure and equilibrated with 1 mM phosphate, pH 7.5. Hydroxylamine-cytochrome *c* reductase activity adhered as a red-colored band in the upper portion of the column. A small amount of 280 mμ absorbing material was removed by the successive elution with 15- to 40-ml volumes of potassium phosphate solution (pH 7.5): 1 mM, 10 mM, 20 mM, 50 mM, 0.1 M, 0.15 M, and 0.2 M. A linear gradient elution procedure with the use of 100-ml volumes of 0.2 to 1.0 M KCl in 1 mM phosphate at pH 7.5 resulted in elution of the enzyme (Fraction 6) between 0.25 and 0.3 M KCl-phosphate with a recovery of 17% of the enzyme units and a specific activity 30-fold that of Fraction 1.

Fraction 6 was dialyzed twice against 400 volumes of 2 mM potassium phosphate (pH 7.5) for a total of 12 hours, diluted to 1:2, and processed through a hydroxylapatite column (1 × 20 cm) as described for the *Nitrosomonas* enzyme to yield an eluate (Fraction 7) containing 17% of the original hydroxylamine-cytochrome *c* reductase units with an over-all 30-fold purification.

As shown in a later section, ferric ions stimulate the enzyme activity 2- to 3-fold in the DEAE-cellulose and hydroxylapatite eluates (Fractions 6 and 7) but not in the crude extract. An adjustment for this stimulation has not been made on the data of Table II. The hydroxylamine-cytochrome *c* reductase of *Nitrosocystis* Fraction 7 had a turnover number of 1,300 (2,600 to 3,900 in the presence of $FeCl_3$) moles of cytochrome *c* reduced per min per mole of enzyme on the assumption of a molecular weight of 100,000 for the enzyme.

Other Enzymatic Activities

In Nitrosomonas Preparations—Although crude extracts of *Nitrosomonas* contained NADH oxidase and cytochrome *c* oxidase activity, the purified preparations (Fractions 5 and 6) had no cytochrome *c* oxidase activity when assayed at pH 9.6, 7.5, or 6.8 with up to 100 times as much enzyme as was used in the standard NH_2OH-cytochrome *c* assay. Succinate and NADPH-cytochrome *c* reductase activities were not present in detectable quantities in the crude extract, nor was NADH- or NADPH-cytochrome *c* reductase or hydroxylamine-NAD, -NADP, -FMN, or -FAD reductase activity observed in the purified preparation. The crude extract and purified enzyme, however, catalyzed the reduction of mammalian cytochrome *c* by hydrazine. Under optimum conditions the maximum rate was one-half the maximum rate of cytochrome *c* reduction by hydroxylamine with the same purified enzyme preparation. The two activities were not additive, for when saturating levels of both hydrazine and hydroxylamine were present in the standard reaction mixture the rate of cytochrome *c* reduction was no greater than that with either substrate alone.

In Nitrosocystis Preparations—Although crude extracts of *Nitrosocystis* contained NADH oxidase and cytochrome *c* oxidase activity, neither of these activities nor hydroxylamine-NAD, -NADP, -FAD, or -FMN reductase (assayed at pH 6.8 and 9.6) was present in the purified *Nitrosocystis* preparation. Hydrazine-cytochrome *c* reductase activity was present but at a level of 5% or less of the hydroxylamine-cytochrome *c* reductase activity. When the *Nitrosocystis* and *Nitrosomonas* purified enzymes were mixed and assayed under conditions of substrate saturation, the rates of hydrazine-cytochrome *c* reductase were additive, indicating that no inhibitor was present in the *Nitrosocystis* preparation. The purified fractions from either organism contained no nitrite- or ammonium sulfate-cytochrome *c* reductase.

Stability of Enzymes—Nitrosomonas whole cells and Fraction 1 packed by centrifugation were stored for over 1 year at −10° with no loss of NH_2OH-cytochrome *c* reductase activity. The enzymatic activity of lyophilized *Nitrosocystis* cells was also stable for several months at −4°. Fraction 6 from both organisms has been stored for 7 months at −10° with essentially no loss in NH_2OH-cytochrome *c* reductase activity. Occasionally, however, Fraction 6 lost activity while stored at 0–4°. When the purified enzyme from either organism was diluted for assay to 10 μg of protein or less per ml and stored overnight at 0–4°, approximately 50% of the activity was lost. Fig. 1 shows the effect of heating a dilute solution of the purified preparation from each organism for 15 min at various temperatures. The *Nitrosomonas* NH_2OH- and NH_2NH_2-cytochrome *c* reductase activities were completely inactivated by heating for 15 min at slightly over 70° and exhibited essentially similar temperature inactivation curves. The purified *Nitrosocystis* NH_2OH-cytochrome *c* reductase was more stable under these conditions, losing 50% of its activity at 70° and 100% at slightly above 80°. Ferric chloride added to the reaction mixture at a final concentration of 5×10^{-5} M partially reversed the inactivation caused by heating. The unheated *Nitrosocystis* preparation was stimulated 15% by $FeCl_3$ in the reaction mixture, whereas the preparation which had been heated at 70° was stimulated 85%. All activity was lost, however, following heating for 15 min at slightly above 80°, and was unaffected by the subsequent inclusion of $FeCl_3$ in the assay procedure.

Nitrosocystis NH_2OH-ferricyanide reductase activity had approximately the same heat sensitivity as the NH_2OH-cytochrome *c* reductase. No activity was lost following heating for 15 min at 70°; half at 80°; and all at 100°.

Cytochrome Components of Nitrosomonas and Nitrosocystis—A comparison of the difference absorption spectra of cell-free extracts (dithionite-treated minus untreated) of *Nitrosomonas* and *Nitrosocystis* indicated the presence of several cytochromes. Absorption maxima at 553, 523, and 420 mμ corresponded to the α, β, and γ peaks of *c* type cytochrome, whereas shoulders at 560 and 530 mμ represented the α and β peaks of a *b* type cytochrome. The maxima at 605 and 445 mμ indicated the α and γ peaks of an *a* type cytochrome.

Curves A and *B* of Fig. 2 show the result of treating the *Nitrosocystis* 100,000 × *g* supernatant solution and pellet with hydroxylamine. As compared with a dithionite-treated preparation (not shown), hydroxylamine reduction resulted in a more prominent shoulder at 560 mμ although the size of all peaks was diminished. *Curves C* and *D* of Fig. 2 show the result of reducing the purified preparations from *Nitrosocystis* and *Nitrosomonas* with hydroxylamine. The ratio of *Nitrosomonas* cytochrome *c* to cytochrome *b* in *Nitrosomonas* Fraction 6 was higher when reduction was accomplished with dithionite than with NH_2OH, indicating that perhaps not all the cytochrome *c* (or a less than proportional amount relative to cytochrome *b*) was reduced by hydroxylamine.

As is summarized in Table III, difference spectra demonstrating the presence of *c*, *b*, and *a* types of cytochrome appeared following the addition of substrate amounts of dithionite, hydroxylamine, or hydrazine in whole cells and in the pellet and supernatant solution resulting from the centrifugation of crude extracts at 100,000 × *g* for 2 hours. Nearly all of the cytochrome *a* was sedimented at 100,000 × *g*, however, and none was present in the purified enzyme. Added ammonium sulfate caused reduction of the *b*, *c*, and *a* types of cytochrome in whole cells but was without effect in cell-free extracts.

Time Course of Enzymatic Reactions and Proportionality of Enzyme Activity with Enzyme Concentration—The time course of cytochrome *c* reduction and concomitant hydroxylamine disappearance catalyzed by the purified *Nitrosocystis* preparation often exhibited two successive linear phases (Fig. 3). The initial rate was constant for the first 10 min and then decreased by a factor of about ½. When hydroxylamine addition to a reaction mixture containing glycine buffer, enzyme, and cytochrome *c* was delayed for 10 min (at which point the rate of cytochrome *c* reduction in a complete reaction mixture containing NH_2OH had decreased to one-half the original rate), cytochrome *c* reduction proceeded at 50% of the normal initial rate. Thus the lowered rate was not ascribed to a limiting quantity of substrate. Neither the use of potassium phosphate (pH 7.5 or 6.8) as buffer in the reaction mixture nor the inclusion of 5×10^{-5} M $FeCl_3$; 5×10^{-4} M FAD; 10^{-4} M $MgCl_2$; 2.5×10^{-4} M reduced glutatione; 0.1% crude DNA, RNA, or crystalline BSA; or doubled hydroxylamine or cytochrome *c* levels protected against the rate decrease. The biphasic time course of hydroxylamine oxidation and cytochrome *c* reduction did not occur with the *Nitrosomonas* enzyme.

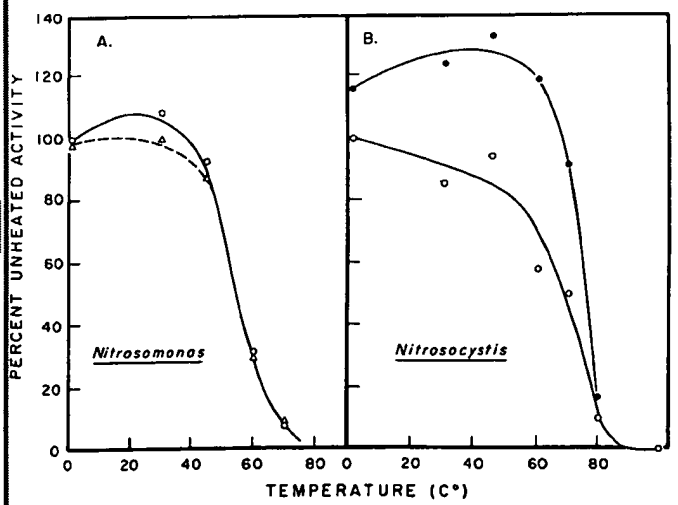

FIG. 1. Effect of temperature on NH_2OH-cytochrome *c* reductase. Aliquots (in 1.5 ml of 0.01 M potassium phosphate, pH 7.5) of purified *Nitrosomonas* (8.6 μg of protein per ml) or *Nitrosocystis* (14 μg of protein per ml) contained in marble-capped Pyrex test tubes (13 × 100 mm) were heated in a water bath for 15 min at the specified temperatures. After having been cooled on ice, each heat-treated *Nitrosomonas* preparation was diluted and assayed for hydroxylamine- (○) and hydrazine- (△) cytochrome *c* reductase activity and each *Nitrosocystis* preparation for hydroxylamine-cytochrome *c* reductase activity in the presence (●) and absence (○) of 5×10^{-5} M $FeCl_3$.

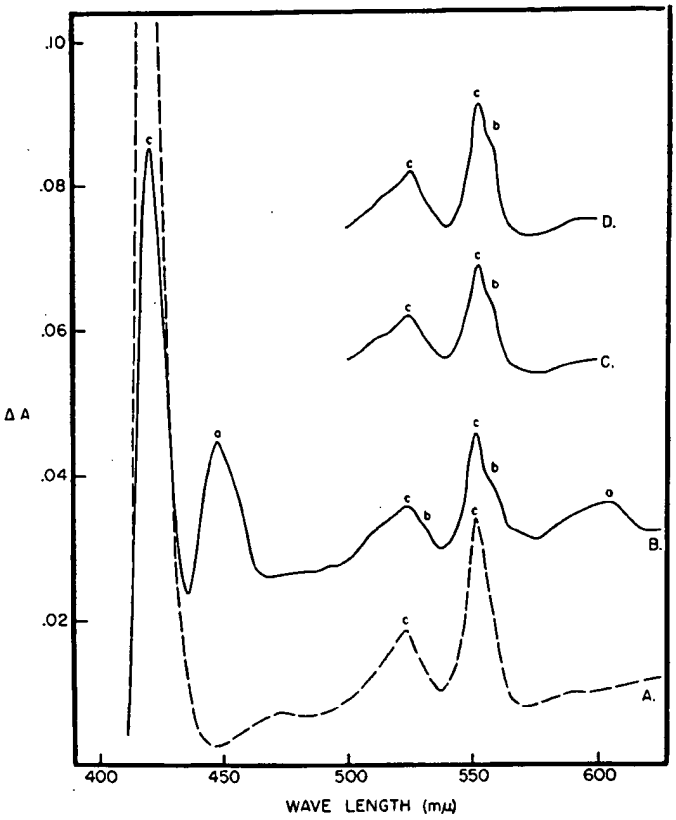

FIG. 2. Hydroxylamine difference spectra of cell-free preparations. Two 0.1-ml samples of extract were diluted with 0.8 ml of 0.1 M phosphate buffer (pH 7.5) in separate quartz cuvettes. With the use of one as a reference, the absorbance of the other was measured over the wave length range of 390 to 650 mμ to establish that the two were identical. Hydroxylamine (0.1 ml of 5×10^{-3} M) was then added to one and 0.1 ml of water to the other, and the resulting difference spectrum was recorded as described in "Experimental Procedure." *Nitrosocystis*-100,000 × *g* supernatant, 3 mg of protein (*A*); -100,000 × *g* pellet, 3 mg of protein (*B*); -hydroxylapatite eluate, 28 μg of protein (*C*); *Nitrosomonas*-hydroxylapatite eluate, 26 μg of protein (*D*). Absorption maxima of cytochromes of the *c*, *b*, and *a* types are identified by the appropriate lower case letters.

pH Optimum—Fig. 4 shows the effect of pH and type of buffer on the cytochrome *c* reductase activities. An optimum between 9.5 and 10 was observed for the three activities studied. The same dependence on pH of enzymatic activity in the crude extracts was exhibited with both cytochrome *c* reduction by hydroxylamine and disappearance of hydroxylamine.

Hydroxylamine and Hydrazine Affinities—Fig. 5 shows the relationship between concentration of hydroxylamine or hy-

TABLE III

Cytochrome components of Nitrosomonas and Nitrosocystis

Difference spectra were measured essentially as described in Fig. 2 on the specified fractions which had been treated with one of the reducing agents listed. The cytochromes were recognized by the characteristic absorption maxima which are given in the text.

Fraction	Reducing agent	Types of cytochrome difference spectra observed
Whole cell suspension	$(NH_4)_2SO_4$, NH_2OH, NH_2NH_2, or dithionite	*c*, *b*, *a*
100,000 × *g* pellet	NH_2OH, NH_2NH_2, or dithionite	*c*, *b*, *a*
100,000 × *g* supernatant solution	NH_2OH, NH_2NH_2 or dithionite	*c*, *b*, trace of *a*
Purified fraction	NH_2OH, NH_2NH_2 or dithionite	*c*, *b*

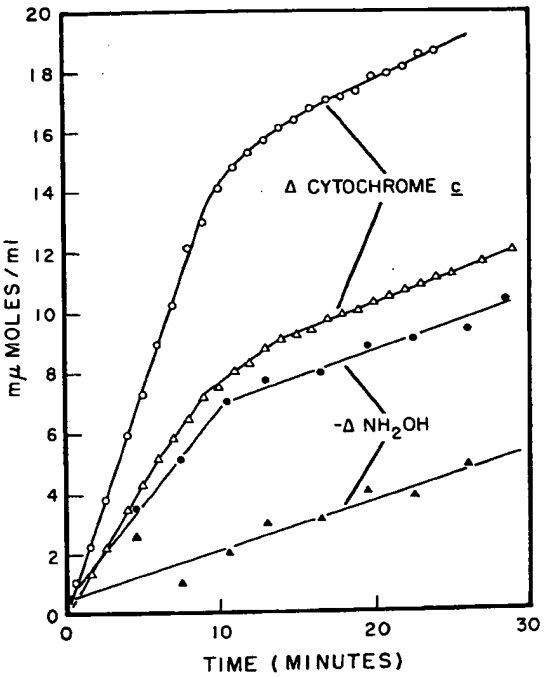

FIG. 3. Cytochrome *c* reduction (○ and △) and hydroxylamine disappearance (● and ▲) catalyzed by the hydroxylapitite eluate (Fraction 7) from *Nitrosocystis* were assayed as described in "Experimental Procedure" with 0.7 μg (△ and ▲) or 1.4 μg (○ and ●) of protein per ml of reaction mixture.

drazine and rate of cytochrome *c* reduction by the enzymes from the two organisms. The K_m values for hydroxylamine and hydrazine in the *Nitrosomonas* enzyme as estimated from Lineweaver-Burke plots of the points from the saturation curves (Fig. 5) were 3.6 and 4 μM, respectively, while the K_m for hydroxylamine in the *Nitrosocystis* enzyme was estimated to be 1.1 μM.

Inhibition of Cytochrome c Reductase Activity by Hydrazine—As shown in Fig. 5, hydrazine at final concentrations greater than 10^{-4} significantly inhibited *Nitrosomonas* NH_2NH_2-cytochrome *c* reductase activity, with 50% inhibition occurring at 10^{-3} M. The rate of hydroxylamine-cytochrome *c* reductase activity (with the use of a final concentration of 2.5×10^{-5} M hydroxylamine) was inhibited by hydrazine concentrations greater than 10^{-5} M, with 50% inhibition occurring at approximately 3×10^{-4} M hydrazine. Hydroxylamine disappearance catalyzed by the *Nitrosomonas* enzyme (in the presence of 5×10^{-5} M hydroxylamine) was inhibited 50% by 10^{-5} M hydrazine and completely by 10^{-3} M hydrazine. Hydroxylamine itself at final concentrations as high as 10^{-3} M did not inhibit the enzyme although 10 to 20% inhibition occurred at a concentration of 0.02 M.

Hydrazine at final concentrations above 10^{-5} M progressively inhibited cytochrome *c* reduction by 10^{-4} M hydroxylamine as catalyzed by the enzyme from *Nitrosocystis* with 50% inhibition occurring at 10^{-4} M hydrazine. When hydroxylamine was excluded from the reaction mixture there was no enzymatic reduction of cytochrome *c* by hydrazine at any of the concentrations employed.

Cytochrome c Affinities—The effect of varying the cytochrome concentration on the rates of the *Nitrosomonas* and *Nitrosocystis* hydroxylamine-cytochrome *c* reductases and the *Nitrosocystis* hydrazine-cytochrome *c* reductase is shown in Fig. 6. At low cytochrome concentrations it was possible to record a linear rate of reduction during the first 60 sec but not during a 2-min period. Therefore the change in absorbance at 550 mμ between 30 and 60 sec, or 0 and 60 sec, as indicated, after the start of the reaction was measured in the assays reported in Fig. 6.

With hydroxylamine as a substrate for the *Nitrosomonas* enzyme a K_m of 10^{-6} M was estimated for cytochrome *c* from a Lineweaver-Burke plot of the data shown in Fig. 6. With hydrazine as the electron donor, the K_m for cytochrome *c* as estimated from the data (not shown) obtained by the standard 2-min assay procedure was 2 to 4×10^{-6} M.

With NH_2OH as the substrate for the *Nitrosocystis* enzyme the estimated K_m for cytochrome *c* was 1 to 1.7×10^{-6} M. Type II cytochrome *c* (60 to 70% pure, Sigma) inhibited the hydroxylamine-cytochrome *c* reductase of both organisms when used at concentrations of 8×10^{-5} M or higher. Table IV summarizes the various substrate affinities described above.

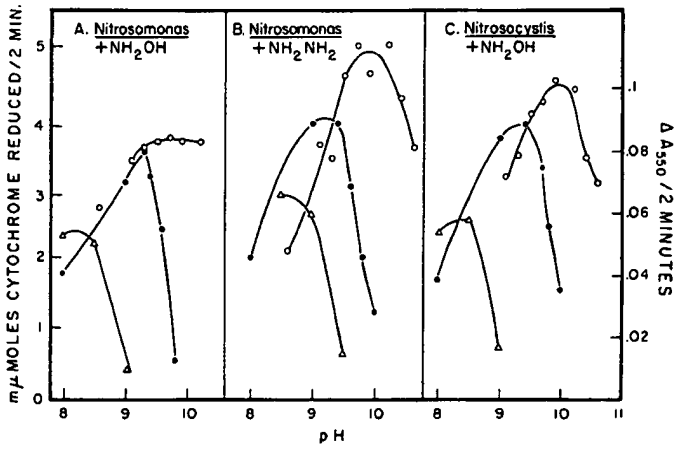

FIG. 4. Effect of buffer and pH on the rate of cytochrome *c* reduction by the hydroxylapatite eluates of *Nitrosocystis* and *Nitrosomonas*. The reaction was run as described in "Experimental Procedure" in 0.05 M sodium carbonate (△), borate (●), or glycine (○) buffer at the indicated pH, with the substrate specified in the figure. The experiments depicted in *A*, *B*, and *C* were performed with 0.33, 0.86, and 0.35 μg, respectively, of the appropriate enzyme per ml of reaction mixture.

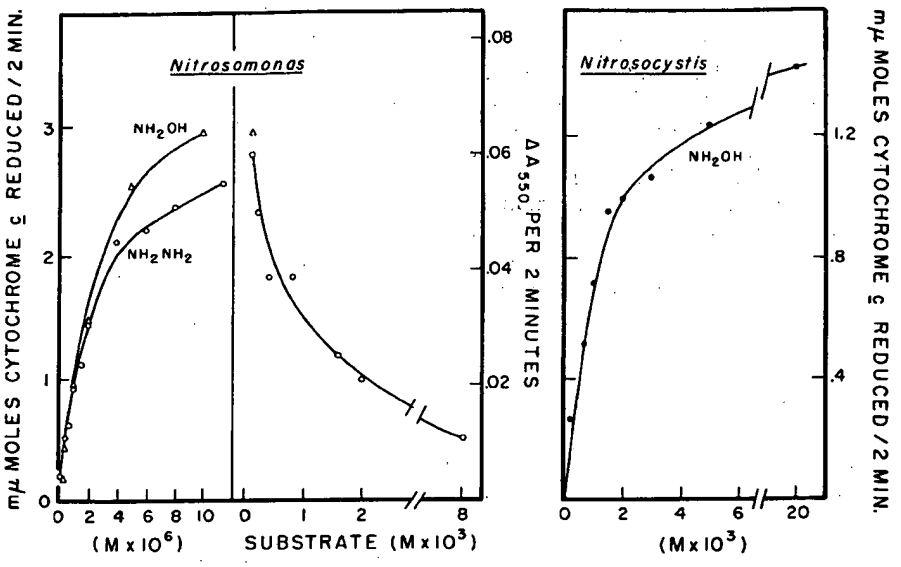

FIG. 5. The effect of hydroxylamine or hydrazine concentrations on the rate of cytochrome *c* reduction catalyzed by hydroxylapatite eluates of *Nitrosomonas* or *Nitrosocystis* (Fractions 6 and 7, respectively). The standard assay procedure was used except that the concentrations of NH_2NH_2 and NH_2OH were varied. The experiments were performed with the following amounts of protein per ml of reaction mixture: △, 0.43 μg of *Nitrosomonas;* ○, 0.43 μg of *Nitrosomonas;* ●, 0.17 μg of *Nitrosocystis*.

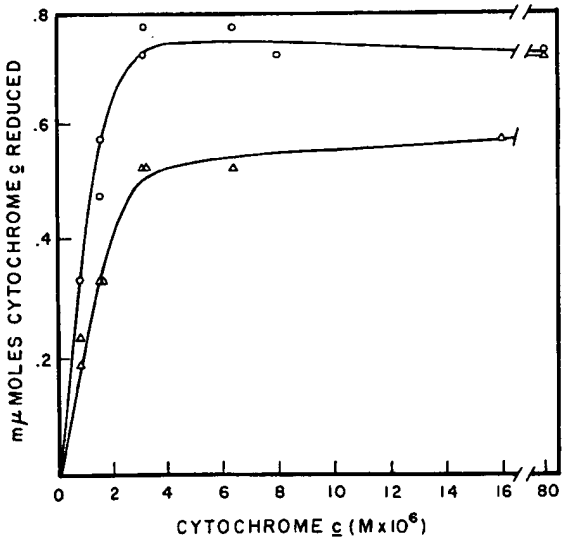

FIG. 6. Effect of cytochrome *c* concentration on the hydroxylamine-cytochrome *c* reductase of *Nitrosomonas* and *Nitrosocystis*. Cytochrome *c* reduction was measured in the standard reaction mixture containing various concentrations of cytochrome *c* as specified. ○, change in A_{550} between 0 and 60 sec after the reaction was started by addition of 0.43 μg of protein of *Nitrosomonas* hydroxylapatite eluate; △, change in A_{550} between 30 and 60 sec after the addition of 0.11 μg of protein of *Nitrosocystis* hydroxylapatite eluate.

TABLE IV

Substrate affinities and inhibition by hydrazine of cytochrome c reductase activities

Source of enzyme	Enzyme activity	Substrate	K_m	Concentration of NH_2NH_2 for 50% inhibition*
			×10⁻⁵ M	M
Nitrosomonas	NH_2OH-cytochrome *c* reductase	NH_2OH	3.6	3×10^{-4} (*a*)
		Cytochrome *c*	1.0	
	NH_2NH_2-cytochrome *c* reductase	NH_2NH_2	4	10^{-3}
		Cytochrome *c*	2–4	
Nitrosocystis	NH_2OH-cytochrome *c* reductase	NH_2OH	1.1	10^{-4} (*b*)
		Cytochrome *c*	1–1.7	

* Inhibition of hydroxylamine-cytochrome *c* reductase was measured in the presence of (*a*) 2.5×10^{-5} M and (*b*) 10^{-4} M NH_2OH.

Oxygen Requirement—The rate of cytochrome *c* reduction by hydroxylamine was not decreased by carrying out the enzymatic reaction anaerobically under the conditions described in "Experimental Procedure" which inhibited nitrite production.

Other Electron Acceptors—Characterization of hydroxylamine-DCI and -ferricyanide reductase activities was performed largely on the *Nitrosocystis* enzyme. Under conditions of substrate saturation in phosphate buffer, pH 7.0, DCI was reduced half as rapidly as cytochrome *c*, whereas the reduction of ferricyanide at pH 8.5 occurred at 20 times the rate of cytochrome *c* reduction at the same pH. Maximum enzymatic DCI reduction by hydroxylamine occurred at 1 to 2×10^{-4} M DCI and half maximum at 3 to 4×10^{-5} M. Ferricyanide was reduced maximally at 7×10^{-4} M and at half that rate at 10^{-4} M. These values are compared with a K_m of 1 to 2×10^{-6} M for cytochrome *c*.

Hydroxylamine was enzymatically oxidized fastest by DCI at a hydroxylamine level of 5×10^{-4} M; oxidation occurred at half that rate at 2×10^{-5} M. With ferricyanide as electron acceptor, corresponding values of 10^{-4} M and 2 to 3×10^{-5} M NH_2OH were observed.

Preliminary experiments indicated that, under conditions allowing extensive hydroxylamine- and hydrazine-cytochrome *c* reductase activity in the presence of the *Nitrosomonas* enzyme, no hydrazine-DCI reductase activity was observed.

Effect of Inhibitors—The NH_2OH-cytochrome *c* reductase

TABLE V

Effect of inhibitors on cytochrome c reductase activity

Inhibitors were dissolved in aqueous solutions at approximately pH 7. In cases where an inhibitor was dissolved in ethanol-water solution, correction was made for inhibition caused by the appropriate final concentration of ethanol. A quantity of purified enzyme was used in the reaction mixture which gave a change in 550 mμ absorbance of 0.1 per 2 min in the absence of inhibitor. Atebrin and HMB inhibitions were assayed in borate buffer, pH 7.5 and 9.6, respectively. Atebrin was preincubated with the enzyme and cytochrome c for 3 min; the other inhibitors were not.

Compound	Final concentration	Inhibition		
		Nitrosomonas enzyme		*Nitrosocystis* enzyme
		NH_2NH_2 as substrate	NH_2OH as substrate	NH_2OH as substrate
	M	%	%	%
Antimycin-A*	5×10^{-3}		89	
	3×10^{-3}			50
Sodium Amytal	0.02		43	44
4-Hydroxy-2-*n*-heptyl quinoline *N*-oxide*	50 μg/ml		0	0
Iodoacetate	10^{-3}		0	0
Sodium arsenate	10^{-2}		0	40†
Sodium arsenite	10^{-2}		0	50†
2,4-Dinitrophenol	$2\text{-}8 \times 10^{-4}$		0–40	50
Atebrin	5×10^{-5}	35	35	0
	10^{-4}	50	50	
	2.5×10^{-4}	60	60	50
	5×10^{-3}	80	88	90
p-Hydroxymercuribenzoate	10^{-2}	78	91	84
	5×10^{-3}	73	73	50
	2×10^{-3}	57	46	10
	2×10^{-4}	16	12	

* Dissolved in an ethanol-water solution.

† These figures represent stimulation rather than inhibition.

activities from the two organisms were closely similar in sensitivity to a number of inhibitors (Table V). Both enzymes were unaffected by the respiratory inhibitors antimycin A, Amytal, and 4-hydroxy-2-*n*-heptyl quinoline *N*-oxide except at levels which were several orders of magnitude above the concentration inhibitory to mammalian electron transport systems. Iodoacetate (10^{-3} M), and arsenate or arsenite (10^{-2} M) were not inhibitory. The inhibition by 2,4-dinitrophenol, which was not consistently reproducible at a given level, was possibly due to a metal-binding effect.

The three cytochrome c reductase activities reported in Table V were inhibited 50% by 1 to 2.5×10^{-4} M atebrin and as much as 90% at higher concentrations. Inhibition was not increased when the enzyme was preincubated with atebrin for 10 min before the addition of hydroxylamine or hydrazine. The addition of either FAD or FMN reversed atebrin inhibition by as much as 80% (Table VI). The rapid nonenzymatic rate of change in 550 mμ absorbance in the presence of flavin prevented the use of high flavin concentrations in attempts to achieve complete reversal. The extent of flavin reversal of atebrin inhibition was dependent on the concentrations of atebrin and flavin; higher atebrin levels required higher flavin levels for reversal. Incubation with FAD or FMN did not stimulate the hydrazine- or hydroxylamine-cytochrome c reductase activity from either organism, and enzymatic reduction of substrate quantities of FAD or FMN by hydrazine or hydroxylamine was not observed under anaerobic conditions.

As Table V indicates, the cytochrome c reductases from the two organisms were 50% inhibited by 2 to 5×10^{-3} M *p*-hydroxymercuribenzoate. Inhibition was not increased by preincubation of the enzyme with HMB for 10 min in the absence of NH_2OH or NH_2NH_2 and occurred in borate buffer (pH 9.6) but not in glycine buffer at the same pH. GSH reversed inhibition of the cytochrome c reductases by HMB by as much as 97% (Table VII), but of itself did not stimulate NH_2NH_2- or NH_2OH-cytochrome c reductase activity.

Effect of Metal-binding Agents—As shown in Table VIII, several metal-binding agents inhibited the NH_2OH- and NH_2NH_2-cytochrome c reductase of *Nitrosomonas* and *Nitrosocystis*, suggesting that a metal ion was involved in the reaction catalyzed by these enzymes. Potassium cyanide, diethyldithiocarbamate, and salicylaldoxime were significantly inhibitory to all three enzymatic activities. Although the three enzymatic activities were inhibited to some extent by diethylydithiocarbamate, the rapid nonenzymatic change in absorbance at 550 mμ in the presence of this compound was a major interfering factor. Both activities of the *Nitrosomonas* preparation were inhibited 50% by 10^{-4} M α,α'-dipyridyl, whereas the *Nitrosocystis* NH_2OH-cytochrome c reductase was uninhibited by the same compound at concentrations as high as 10^{-3} M. Allylthiourea at 2×10^{-3} M and 10^{-2} M inhibited the *Nitrosomonas* hydroxylamine-cytochrome c reductase 34 and 79%, respectively. Inhibition by azide, EDTA, and orthophenanthroline occurred only at inordinately high concentrations (approximately 10^{-2} M), if at all.

Stimulation by Ferric Ions—Ferric ions consistently stimulated the *Nitrosocystis* hydroxylamine-cytochrome c reductase activity 2- to 4-fold (as measured by rate of hydroxylamine disappearance and rate of cytochrome c reduction). None of the other enzymatic activities was affected. Stimulation of the *Nitrosocystis* hydroxylamine-cytochrome c reductase by iron was greatest when the enzyme had been dialyzed or had been standing at 0° for several hours following dilution, and it was highly specific for ferric ions. Neither Co^{++}, Zn^{++}, Mn^{++}, Mg^{++}, Ca^{++}, nor $MoO_4^{=}$ was effective at a concentration of 10^{-4} M (Table IX). The slight enhancement of *Nitrosomonas* hydrazine-cytochrome c reductase activity and *Nitrosocystis* hydroxylamine-cytochrome c reductase activity by cupric ions and of *Nitrosomonas* hydroxylamine-cytochrome c reductase by ferric ions occurred occasionally and was not reproducible. In a preliminary experiment with high levels of enzyme, ferric ion was not found to stimulate the hydrazine-cytochrome c reductase activity from *Nitrosocystis*.

Ferric chloride gave a maximum stimulation (300 to 400%) of *Nitrosocystis* hydroxylamine-cytochrome c reductase at 5×10^{-4} M with half maximum stimulation occurring at 10^{-4} M (Fig. 7). No stimulation occurred at pH 7.5, whereas 20 to 50% stimulation was effected between pH 8 and 8.5, with maximum stimulation at pH 9.5. The possibility that ferric iron was acting as an electron carrier prompted attempts to detect ferrous iron-cytochrome c reductase and hydroxylamine-ferric iron reductase activities. No iron-cytochrome c reductase activity was observed, although the nonenzymatic reduction of cytochrome c

TABLE VI

Reversal by flavins of atebrin inhibition of cytochrome c reduction

A standard reaction mixture (with borate buffer, pH 7.5, since atebrin and flavin precipitated at a higher pH) was preincubated for 3 min in the presence of the indicated concentration of atebrin and the absence of substrate (NH_2NH_2 or NH_2OH). Substrate and flavin were then added, and the rate of cytochrome *c* reduction was measured in the standard manner. The rate for the mixture treated in this way was as reported in the column headed "Reversed." A second reaction mixture (column headed "Inhibited") was simultaneously treated in an identical manner except that no flavin was added. A third reaction mixture (column headed "Control") was preincubated in the absence of atebrin and assayed in the standard manner without flavin. Each rate value was corrected for the change in absorbance occurring in an identically treated reaction mixture which contained no enzyme.

Source of enzyme	Substrate	Atebrin concentration	Enzyme activity			Type of flavin used	Flavin concentration
			Control	Inhibited	Reversed		
		M	ΔA_{430}/2 min				M
Nitrosomonas	NH_2NH_2	2.5×10^{-4}	0.095	0.031	0.048	FAD	10^{-3}
		2.5×10^{-4}	0.076	0.025	0.059	FMN	10^{-3}
	NH_2OH	10^{-3}	0.140	0.031	0.077	FAD	2×10^{-3}
		10^{-3}	0.140	0.035	0.081	FMN	2×10^{-3}
Nitrosocystis	NH_2OH	10^{-3}	0.143	0.013	0.068	FAD	2×10^{-3}
		10^{-3}	0.062	0.014	0.046	FMN	2×10^{-3}

TABLE VII

GSH reversal of HMB inhibition of cytochrome c reductase

The experiments were run essentially as described in Table VI (with borate buffer, pH 9.6) except that HMB and GSH were used in place of atebrin and flavin, respectively.

Enzyme	Substrate	HMB concentration	Enzyme activity			GSH concentration
			Control	Inhibited	Reversed	
		M	ΔA_{340}/2 min			M
Nitroso-monas	NH_2OH	5×10^{-3}	0.097	0.029	0.087	5×10^{-3}
	NH_2NH_2	7×10^{-3}	0.105	0.019	0.075	7×10^{-3}
Nitroso-cystis	NH_2OH	8×10^{-3}	0.134	0.021	0.130	8×10^{-3}

by ferrous ions (10^{-4} M) was rapid enough to have possibly obscured any enzymatic activity. Moreover reproducible enzymatic hydroxylamine disappearance could not be detected in a standard reaction mixture lacking cytochrome *c* but containing in its place $FeCl_3$ (final concentration, 10^{-3} M) as an electron acceptor and α,α'-dipyridyl to form a complex with ferrous ion.

Ferric chloride did not stimulate the enzymatic reduction of DCI or ferricyanide by hydroxylamine.

Stoichiometry of Reaction—The data of Table X show that in the presence of the *Nitrosomonas* enzyme an average of 2.6 moles of mammalian cytochrome *c* were reduced per mole of hydroxylamine oxidized at pH 9.6, whereas the ratio fell to 1.5 at pH 6.8. Consistent measurements of the quantity of hydroxylamine oxidized were made difficult because (*a*) the intensity of color development per mole of hydroxylamine was found to vary in a standard hydroxylamine curve determined with each experiment and (*b*) hydroxylamine disappeared nonenzymatically at a rate comprising as much as 30% of the total hydroxylamine disappearance.

With the purified *Nitrosocystis* preparation the ratio of cytochrome *c* reduced to hydroxylamine oxidized (cytochrome

TABLE VIII

Inhibition of cytochrome c reductase activity by metal-binding agents

The rate of cytochrome *c* reduction assayed in the standard manner was compared with the rate in identical reaction mixtures containing 0.3 to 0.7 μg of enzyme and specified levels of inhibitor. In cases in which an inhibitor was dissolved in aqueous ethanol solution, correction was made for the inhibitory effect of the appropriate final concentration of ethanol.

Compound	Concentration	Inhibition		
		Nitrosomonas enzyme		*Nitroso-cystis* enzyme
		NH_2NH_2	NH_2OH	NH_2OH
	M	%	%	%
KCN	2×10^{-5}	44	49	57
α,α'-Dipyridyl	10^{-3}	90	90	0
	10^{-4}	48	65	
Diethyldithiocarbamate	10^{-4}	I*	I	I
Salicylaldoxime	6×10^{-3}	47	41	65
Allylthiourea	10^{-3}		79	
	2×10^{-3}		34	
NaN_3†	0.02	0	0	0
EDTA	0.015			50
Orthophenanthroline†	0.02	95	78	42

* I, inhibitory.

† Certain inhibitors were incubated with the enzyme for 3 min before the substrate was added. Inhibitors were in aqueous solutions at approximately pH 7.

TABLE IX

Effect of metal ions on cytochrome c reductase

Cytochrome *c* reduction was assayed by the standard procedure except that the reaction mixture containing 0.3 to 0.6 μg of enzyme was incubated for 3 to 5 min with 10^{-4} M metal ion before addition of NH_2NH_2 or NH_2OH to start the reaction. The rapid nonenzymatic reduction of cytochrome *c* by ferrous ions precluded the possibility of testing their effect on cytochrome *c* reductase.

Compound added	*Nitrosomonas*		*Nitrosocystis*
	NH_2OH-cytochrome *c* reductase	NH_2NH_2-cytochrome *c* reductase	NH_2NH_2-cytochrome *c* reductase
	%	%	%
$FeCl_3$	0	−3	220
$CuCl_2$	−21	15	21
$CoCl_2$	−25	−17	0
$ZnCl_2$	−5	−18	−4
$MgCl_2$	2	−10	14
$MnCl_2$	−8	−51	7
$CaCl_2$	11	−35	5
Na_2MoO_4	−12	−20	22

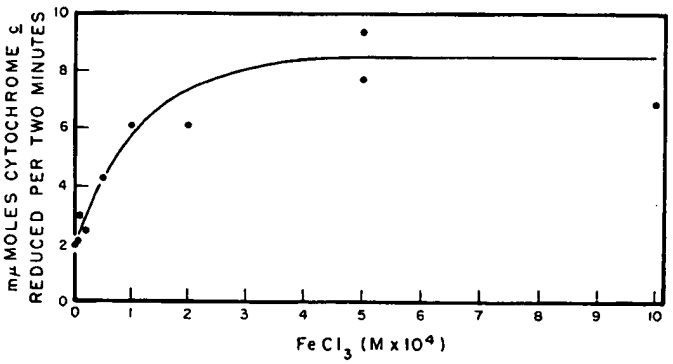

FIG. 7. Effect of added $FeCl_3$ on *Nitrosocystis* hydroxylamine-cytochrome *c* reductase. Enzyme activity of 0.28 μg of protein of hydroxylapatite eluate per ml of reaction mixture was measured by the standard procedure except that the $FeCl_3$ concentration was varied.

TABLE X

Stoichiometry of Nitrosomonas hydroxylamine-cytochrome c reductase

The rates of hydroxylamine disappearance and cytochrome *c* reduction were measured as shown in Fig. 3 except that phosphate buffer, pH 6.8, was used where indicated in place of glycine, pH 9.6. The changes in hydroxylamine and cytochrome *c* during 10 to 20 min of a linear reaction catalyzed by *Nitrosomonas* fractions 5 or 6 were corrected for the appropriate nonenzymatic changes and averaged.

No. of experiments	pH	Average time of reaction	Average amount of enzyme	Average nonenzymatic decrease in NH_2OH	Average enzymatic decrease in NH_2OH	Average enzymatic cytochrome *c* reduction	Average ratio of cytochrome *c* reduced to decrease in NH_2OH
		min	*μg/ml*	*mμmoles*			
4	9.6	13.5	0.64	3.53	10.2	26.6	2.6
2	6.8	17	3.0	5.75	24.3	32.4	1.6

TABLE XI

Stoichiometry of Nitrosocystis hydroxylamine-cytochrome c reductase

The rate of hydroxylamine disappearance, cytochrome *c* reduction, and nitrite production were measured as described in Table X. The changes in hydroxylamine, cytochrome *c*, and nitrite during 10 to 15 min of a linear reaction catalyzed by *Nitrosocystis* Fraction 6 were corrected for the appropriate nonenzymatic changes, adjusted to a standard 20-min time period, and averaged. The changes were also measured simultaneously in a reaction mixture containing 10^{-4} M $FeCl_3$ as indicated.

No. of experiments	$FeCl_3$	Average amount of enzyme	Enzyme activities			Ratio of activities	
			Cytochrome *c* reduction	Decrease in NH_2OH	Change in NO_2^-	Cytochrome *c* reduced to decrease in NH_2OH	Change in NO_2^- to decrease in NH_2OH
		μg/ml	*mμmoles/ml/20 min*				
5	−	0.62	29	13	0.82	2.1	0.065
5	+	0.44	57	22	3.2	2.6	0.145

c:NH_2OH) was approximately 2 (Table XI); it was 2.6 in the presence of 10^{-4} M $FeCl_3$. The cytochrome *c*:NH_2OH ratio decreased with aging of diluted enzyme, and, in those instances in which cytochrome *c* reduction and hydroxylamine oxidation took place in two linear phases (Fig. 3), the cytochrome *c*:NH_2OH ratio was lower in the second phase than in the first. In some cases cytochrome *c* reduction decreased to less than half its initial rate with almost no concomitant decrease in the rate of hydroxylamine disappearance. It appears that for each mole of hydroxylamine oxidized 1 to 3 moles of cytochrome *c* can be reduced, depending on the state of the preparation.

Product of Enzymatic Reaction—With either *Nitrosomonas* or *Nitrosocystis* purified NH_2OH-cytochrome *c* reductase the only observed product of hydroxylamine oxidation was nitrite. The latter, however, accounted for only 5 to 10% of the hydroxylamine which disappeared (Table XI). It is worthy of note that the inclusion of $FeCl_3$ in the *Nitrosocystis* reaction mixture markedly increased the quantity of nitrite produced as well as the ratio of nitrite formed to hydroxylamine oxidized (Table XI). The product of NH_2NH_2 oxidation by *Nitrosomonas* purified enzyme was apparently neither hydroxylamine nor nitrite. *Nitrosomonas* Fraction 6 in a standard NH_2OH-cytochrome *c* reductase reaction mixture produced 1.25 mμmoles of nitrite from hydroxylamine while in a standard NH_2NH_2-cytochrome *c* reductase reaction mixture no detectable nitrite was produced by 14 times as much of the same enzyme.

DISCUSSION

As summarized in Table XII, the properties of the hydroxylamine-cytochrome *c* reductases from *Nitrosomonas* and *Nitrosocystis* are similar in many respects. Although the halophilic *Nitrosocystis*, in contrast to the nonhalophilic *Nitrosomonas*, will grow only in a medium which includes approximately 3% sodium chloride, the purified enzyme from either organism is neither inhibited nor stimulated by sodium chloride. The hydroxylamine-cytochrome *c* reductase from *Nitrosomonas* and *Nitrosocystis* were almost identical in chromatographic behavior. They adhered at pH 7.5 to DEAE-cellulose and hydroxylapatite and exhibited similar elution properties. Both activities were

TABLE XII

Comparison of Nitrosomonas NH_2OH-cytochrome c reductase with Nitrosocystis NH_2OH-cytochrome c reductase

Properties	*Nitrosomonas* NH_2OH-cytochrome *c* reductase	*Nitrosocystis* NH_2OH-cytochrome *c* reductase
Physical state	Mol. wt. approximately 1–2 × 10^5 estimated from behavior on Sephadex	Same
	In supernatant solution of sonic extracts centrifuged for 2 hr at 100,000 × *g*	Same
	Adheres to DEAE-cellulose at pH 7.5, eluted by 0.25 M KCl	Same, elutes at approximately 0.3 M KCl
	Adheres to hydroxylapatite at pH 7.5 eluted by 0.2–0.3 M potassium phosphate	Same
Heat sensitivity	Stable to heating for 15 min at 50°; all activity lost at 70°	Stable to heating for 15 min at 70°; all activity lost at 85°
Components of electron transport chain	Cytochrome *b* and *c* and possibly flavin	Same
pH optimum	9.5–10 in glycine buffer	Same
K_m for NH_2OH	3.6×10^{-6} M	1.1×10^{-6} M
K_m for cytochrome *c*	10^{-6} M	$1–1.7 \times 10^{-6}$ M
Moles of cytochrome *c* reduced per min per mole of enzyme	2,850	1,300 (2,600–3,900 with ferric ions)
Other electron acceptors	DCI, ferricyanide (only two tried)	Same
Inhibitors	Insensitive to electron transport inhibitors antimycin-A, Amytal, 4-hydroxy-2-*n*-heptyl quinoline *N*-oxide, iodoacetate, arsenite, and arsenate	Same
	Sensitive to atebrin, HMB, KCN, α,α'-dipyridyl, diethyldithiocarbamate, salicylaldoxime, allylthiourea	Same
Metal ion stimulators	None	Ferric ion specifically activates 2- to 4-fold
Stoichiometry of reaction (ratio of cytochrome *c* reduced to NH_2OH oxidized)	2.6	2.1 (2.6 with ferric ion)
Product	Unidentified intermediate of probable oxidation state between NH_2OH and NO_2^-; little or no NO_2^-	Same
NH_2NH_2-cytochrome *c* reductase activity	V_{max} 50% of NH_2OH-cytochrome *c* reductase	Almost entirely absent, V_{max} 0–5% of NH_2OH-cytochrome *c* reductase

eluted from Sephadex G-200 in a manner characteristic of a molecule with a molecular weight of 100,000 to 200,000.

Because of (*a*) its large size, (*b*) the association of at least two types of cytochrome (*b* and *c* types), (*c*) the apparent involvement of flavin and iron, and (*d*) the obvious similarity, from a comparative biochemical viewpoint, of this system to other established electron transport chains such as DPNH- and succinate-cytochrome *c* reductases (see, for example, Reference 16), it seems likely that each enzyme is in reality a complex of associated proteins, activators, and possibly lipids rather than a single simple protein. Difference spectra of whole cells or crude extracts oxidizing NH_2OH indicate the involvement of cytochromes of the *b*, *c*, and *a* types. The terminal cytochrome oxidase (cytochrome *a*), however, is apparently separated during the course of purification of the NH_2OH-cytochrome *c* reductase. It can be speculated that the natural sequence of electron transport in NH_2OH-cytochrome *c* reductase involves the following:

$$NH_2OH \rightarrow \text{flavin } (Fe^{+++}) \rightarrow \text{cytochrome } b \rightarrow \text{cytochrome } c$$

The present evidence for the involvement of flavin is the inhibition of enzymatic activity by the flavin analogue atebrin and the reversal of this effect by either FMN or FAD. A similar effect was reported by Falcone *et al.* (5) for hydroxylamine reduction of cytochrome *c* catalyzed by crude extracts of *Nitrosomonas*. Treatment of the partially purified enzyme preparation used in the present investigation with acid-ammonium sulfate in order to dissociate flavin from apoenzyme failed to result in a flavin requirement by the NH_2OH-cytochrome *c* reductase. Although

Falcone *et al.* (5) reported that spectral changes characteristic of flavin reduction can be induced by adding hydroxylamine to a preparation similar to the crude fractions used in this study, it is not certain that similar spectral changes observed in the present study were due to flavin reduction. Attempts to detect the presence of flavin in the purified enzyme by fluorescence assay were not successful. The unusual stimulation by atebrin of hydroxylamine-DCI activity and the reversal of this effect by flavin are probably explained in terms of nonenzymatic reactions giving absorbance changes at 600 mμ.

The *Nitrosocystis* enzyme is stimulated specifically by ferric iron, especially following dialysis, dilution, or aging. Although the same effect could not be demonstrated with the *Nitrosomonas* enzyme, it is possible that iron is also involved in the hydroxylamine-cytochrome *c* reductase from this organism but is more tightly bound. Iron might function in these systems (*a*) as an electron carrier undergoing alternate oxidation and reduction, (*b*) by stabilization of the structural organization of the complex, (*c*) by promotion of substrate or cofactor binding, or (*d*) in some other indirect manner. It should be noted that at pH 9.6, which was the optimum pH for ferric chloride stimulation, most or all ferric iron was found as the insoluble ferric hydroxide. In addition to stimulating the *Nitrosocystis* NH_2OH-cytochrome *c* reductase, iron stimulated nitrite production in both organisms.

It is quite clear that nitrite is not the immediate product of enzymatic hydroxylamine oxidation since the purified hydroxylamine cytochrome *c* reductase preparations used in the present study resulted in little or no nitrite formation whereas substantial quantities of hydroxylamine disappeared. The direct product of hydroxylamine oxidation catalyzed by NH_2OH-cytochrome *c* reductase is not known but is presumably a substance with nitrogen in an oxidation state between that of hydroxylamine (−1) and nitrite (+3).

If the hydroxylamine dehydrogenase reaction involves the transfer from hydroxylamine to cytochrome *c* of either 1 or 2 electrons, then the nitrogen atom in the resulting product would be expected to be in the zero oxidation state (as in N_2) or +1 state (as in N_2O, $H_2N_2O_2$, or HNO), respectively. The +1 state appears more probable in view of the value of 2 moles of cytochrome reduced per mole of hydroxylamine oxidized reported by Falcone *et al.* (5). That ratio is based on the assumption that all of the hydroxylamine in their reaction mixture was enzymatically oxidized. In addition, they report that N_2O, which is a spontaneous breakdown product of $H_2N_2O_2$ and HNO, is liberated by the reaction in place of nitrite.

It is possible that an intermediate of the +1 state can be further oxidized either (*a*) by the same enzyme, (*b*) by a separate enzyme, or (*c*) nonenzymatically to the +2 oxidation state of nitrogen (perhaps to NO or N_2O_2). In fact, the presently observed stoichiometry ranging from 2.1 to 2.6 moles of cytochrome *c* reduced per mole of NH_2OH oxidized could indicate that hydroxylamine can be oxidized by these extracts to a compound containing nitrogen in either the +1 or +2 oxidation state. The above values are subject to the limitations due to the difficulty encountered in measuring the enzymatic rate of hydroxylamine disappearance. The possible involvement of NO as an intermediate compound in hydroxylamine oxidation to nitrite is consistent with the demonstration by Anderson (6) of cytochrome *c*-stimulated uptake of NO in the presence of a particulate *Nitrosomonas* extract with ferricyanide as an electron acceptor. It is also interesting that the ratio of cytochrome *c* to NH_2OH varied among experiments and occasionally decreased to a value of less than 2 with aging of the preparation.

Aleem and Lees (17) have suggested that the biological conversion of hydroxylamine to nitrite takes place via a nitroxyl intermediate. It was then proposed that nitroxyl combines with nitrous acid to form "nitrohydroxylamine," which could be subsequently ouidized to nitrite by molecular oxygen.

The pH optimum (9.5 to 10) for hydroxylamine-cytochrome *c* reductase is higher than that (8.5) of a similar system in *Nitrosomonas* reported by Aleem and Lees (17) due, most likely, to their use only of carbonate buffer at higher pH values, which is shown here to be inhibitory. Nicholas and Jones (4) reported that the pH optimum for nitrite production from hydroxylamine by extracts of *Nitrosomonas* was between 7.0 and 8.6, and Engel and Alexander (18) observed approximately the same pH optimum for hydroxylamine oxidation to nitrite by resting *Nitrosomonas* cells.

The *Nitrosomonas* hydrazine-cytochrome *c* reductase activity is the same as that observed by Nicholas and Jones (4). It resulted in the production of neither hydroxylamine nor nitrite and probably accounts for the inhibitory effect of hydrazine on NO_2^- production from hydroxylamine by intact cells first reported by Hofman and Lees (2). The hydrazine- and hydroxylamine-cytochrome *c* reductase activities are similar in several properties, including heat stability, pH optimum, substrate affinity, and sensitivity to a number of inhibitors. This information, together with results of a number of experiments reported in the present paper, suggests that hydrazine possibly interacts with cytochrome *c* reductase in essentially two ways: (*a*) at final concentrations of 10^{-6} to 10^{-5} M as an electron donor and competitive inhibitor of hydroxylamine oxidation and (*b*) at final concentrations of 10^{-4} to 10^{-3} M as a noncompetitive inhibitor, (perhaps as a metal- or carbonyl-binding agent) of the enzyme.

The present data are not sufficient to establish the positions at which DCI or ferricyanide act to accept electrons in the electron transport chain. The *Nitrosocystis* DCI and ferricyanide reductase activities differ from the cytochrome reductase in that they are not stimulated by Fe^{+++}, suggesting that both electron acceptors act at some point in the pathway prior to the involvement of iron.

While this work was in progress, several independent reports on similar systems in *Nitrosomonas* were published (5, 17). The preparation of Aleem and Lees (17) differs from the purified enzyme described here in that (*a*) the specific activity is only 5% as great, (*b*) the ability to produce nitrite from hydroxylamine was retained, (*c*) cytochrome *c* reduction was inhibited by 5×10^{-4} M hydroxylamine, completely by 0.01 M, and (*d*) the K_m (1.3×10^{-4} M) for cytochrome *c* was considerably higher than that determined in the present work (1 to 2×10^{-6} M).

Falcone *et al.* (5) have reported a particulate nitrite-producing system containing NH_2OH-cytochrome *c* reductase activity as well as a NADPH-, and succinate-cytochrome *c* reductase and cytochrome oxidase activity.

SUMMARY

Hydroxylamine-cytochrome *c* reductases from *Nitrosomonas* and *Nitrosocystis* were purified from the supernatant solution resulting from a 2-hour centrifugation at 100,000 × *g* of extracts prepared by sonic oscillation. The *Nitrosomonas* enzyme was purified 14-fold with approximately 25% recovery by adsorption

on calcium phosphate gel, diethylaminoethyl cellulose chromatography, ammonium sulfate precipitation, and chromatography on Sephadex G-200 and hydroxylapatite. The *Nitrosocystis* enzyme was purified 30-fold with 17% recovery by fractionation with heat treatment; pH and ammonium sulfate precipitation; and chromatography on Sephadex G-200, DEAE-cellulose and hydroxylapatite. The *Nitrosomonas* and *Nitrosocystis* preparations reduced 2,850 and 1,300 moles of cytochrome *c* per min per mole of enzyme, respectively.

The purified enzyme contained neither reduced nicotinamide adenine dinucleotide-, NADPH-, nitrite-, ammonia-, nor succinate-cytochrome *c* reductase activity. Cytochrome *c*, 2,6-dichlorophenolindophenol, and ferricyanide were suitable electron acceptors while NAD, NADP, flavin mononucleotide, and flavin adenine dinucleotide were not enzymatically reduced by hydroxylamine. The *Nitrosomonas* enzyme contained hydrazine-cytochrome *c* reductase activity which was similar to the NH_2OH-cytochrome *c* reductase in almost all its properties. Enzymatic nitrite production from hydroxylamine accounted for only 10% or less of the hydroxylamine consumed. Neither nitrite nor hydroxylamine was an enzymatic product of hydrazine oxidation.

Although reduced spectra of cytochromes of the *b*, *c*, and *a* type appeared when whole cells or sonic extracts were treated with dithionite, hydrazine, or hydroxylamine, only cytochromes *b* and *c* appeared when the partially purified fractions were treated in the same way. Both enzymes are stable for months at −4° and are completely inactivated by heating at 80–85° for 15 min. Ferric ions completely restored the activity of *Nitrosocystis* enzyme, which had been 50% inactivated at 70°. Both enzymes showed a fairly narrow pH optimum (pH 9.5 to 10 in borate or glycine buffer) and were inhibited by carbonate above pH 8.5.

Hydroxylamine had a K_m of 3.6×10^{-6} M and 1×10^{-6} M for the NH_2OH-cytochrome *c* reductase of *Nitrosomonas* and *Nitrosocystis*, respectively, while the corresponding K_m values for cytochrome *c* were 10^{-6} M and 1.7×10^{-6} M. For the *Nitrosomonas* hydrazine-cytochrome *c* reductase, hydrazine and cytochrome *c* had K_m values of 4×10^{-6} M and 2 to 4×10^{-6} M, respectively. Hydrazine inhibited NH_2OH-cytochrome *c* reductase of *Nitrosomonas* and *Nitrosocystis* 50% at 1 to 3×10^{-4} M and inhibited *Nitrosomonas* NH_2NH_2-cytochrome *c* reductase 50% at 10^{-3} M. Hydroxylamine inhibited the hydroxylamine-cytochrome *c* reductase from either organism 10 to 20% at 0.02 M.

Neither of the hydroxylamine-cytochrome *c* reductases required the presence of oxygen. The enzymes were inhibited by antimycin-A, Amytal, 4 hydroxy-2-*n*-heptyl quinoline *N*-oxide, iodoacetate, arsenite, and arsenate only at high levels, if at all. Inhibition of the two enzymes and *Nitrosomonas* NH_2NH_2-cytochrome *c* reductase by either atebrin or *p*-hydroxymercuribenzoate was reversed by flavin (FAD or FMN) or glutathione, respectively. None of the enzymatic activities was stimulated by flavin or glutathione. Several metal-binding agents, including potassium cyanide, α,α'-dipyridyl, diethyldithiocarbamate, and salicylaldoxime, inhibited both NH_2OH-cytochrome *c* reductases.

Nitrosocystis hydroxylamine-cytochrome *c* reductase was stimulated specifically by ferric ions and not by cobalt, zinc, manganese, magnesium copper, calcium, or molybdate ions. The stimulation was greatest at a concentration of 4×10^{-4} M $FeCl_3$ and was favored when the pH was in the vicinity of 9.6.

For each mole of hydroxylamine oxidized by the *Nitrosomonas* NH_2OH-cytochrome *c* reductase 2.6 moles of cytochrome *c* were reduced enzymatically. The ratio of cytochrome *c* to NH_2OH with *Nitrosocystis* was 2.1 and 2.6 in the absence and presence of ferric ion, respectively.

The transfer of electrons from NH_2OH to oxygen is postulated to occur via NH_2OH-cytochrome *c* reductase and cytochrome oxidase as follows.

$$NH_2OH \rightarrow \text{flavin } (Fe^{+++}) \rightarrow \text{cytochrome } b \rightarrow \text{cytochrome } c \rightarrow \text{cytochrome } a \rightarrow O_2$$

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APPENDIX C

RESEARCH ARTICLES

INTERNATIONAL MICROBIOLOGY (2005) 8:103–110
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INTERNATIONAL MICROBIOLOGY

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Polygenic analysis of ammonia-oxidizing bacteria using 16S rDNA, *amoA*, and *amoB* genes

Summary. Finding a unique molecular marker capable of quickly providing rigorous and useful phylogenetic information would facilitate assessing the diversity of ammonia-oxidizing bacteria in environmental samples. Since only one of several available markers can be used at a time in these kinds of studies, the 16S rDNA, *amoA* and *amoB* genes were evaluated individually and then compared in order to identify the one that best fits the information provided by the composite dataset. Distance-based neighbor-joining and maximum parsimony trees generated using the sequences of the three mentioned genes were analyzed with respect to the combined polygenic trees. Maximum parsimony trees were found to be more accurate than distance-based ones, and the polygenic topology was shown to best fit the information contained in the sequences. However, the taxonomic and phylogenetic information provided by the three markers separately was also valid. Therefore, either of the functional markers (*amoA* or *amoB*) can be used to trace ammonia oxidizers in environmental studies in which only one gene can be targeted. **[Int Microbiol** 2005; 8(2):103-110]

Key words: ammonia-oxidizing bacteria · 16S rDNA · *amoA* · *amoB* · polygenic analysis

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Introduction

Environmental and biotechnological interest in ammonia-oxidizing bacteria (AOB) has increased tremendously in recent years. However, their slow growth and the difficulty to be cultured have necessitated the development of a variety of culture-independent techniques for carrying out ecologic and taxonomic studies [18,19,21,28,48,53,54]. These techniques include the use of 16S rDNA and protein-encoding genes to characterize natural AOB populations [4,7,25,42,46] and to analyze their taxonomic and phylogenetic features [1,2,6,37,38]. Nonetheless, although 16S rDNA sequences are suitable for providing a comprehensive long-term evolutionary view of prokaryotic taxonomy, they fail to discriminate among close relatives, such as species within a given group or genus [39]. In addition, considerable variability can be found among organisms with almost identical 16S rDNA genes [3]. Thus, while 16S rDNA has proven useful in the discrimination between nitrosococci and nitrosomonads [5,55], the outcome is confusing when examining a single genus, such as *Nitrosospira* [41]. For this reason, protein-encoding genes, such as *amoA*, have been added to the collection of comparative tools used by taxonomists and molecular ecologists for diversity studies [14,46]. Gene *amoA* codes for the active site of ammonia monooxygenase [30], and it has been extensively used for the detection and study of ammonia oxidizers, particularly in natural environments [1,15,21]. According to Rotthauwe et al. [41], *amoA* is more useful at a fine-scale than 16S rDNA. By contrast, Ludwig and Schleifer [27] stated that the 16S rDNA gene is the best marker to infer phylogenetic relationships, since the topologies derived from 16S rDNA are in accordance with those obtained using markers with rather diverse functions. This has been recently supported by Purkhold et al. [38], who showed higher resolution using 16S rDNA than *amoA* within the tradi-

tional classification. Recently, *amoB* has been shown to be a suitable molecular marker for the study of AOB, as it has a high capacity of resolution within genera. In addition, its phylogeny is highly consistent with the current taxonomic outline [6].

The reconstruction of phylogenetic relationships between closely related species requires the use of markers with significant mutation rates; however, the accumulation of recurrent mutations results in the incorporation of large amounts of mutational homoplasy into the molecular data [47]. In addition, when mutations occur repeatedly at the same site, those that occurred later mask the previous ones, rendering the sequences useless for phylogenetic purposes. This phenomenon, known by geneticists as substitution saturation, should be taken into account before proceeding with any type of phylogenetic analysis [12]. It should also be noted that polymorphisms detected in the sequences of a given population reveal not only the mutations experienced by the ancestors but also the consequences of evolutionary forces, such as genetic drift and natural selection. It is therefore essential to check whether the molecular dataset has been affected by evolutionary pressures, especially since the neutral theory has become the standard null hypothesis in the study of molecular evolution [13,22].

In the present work, the 16S rDNA, *amoA*, and *amoB* genes were used to determine whether or not a polygenic or single-marker analysis was more suitable for taxonomic studies of AOB. These three markers were evaluated independently in a panel of genetic tests to compare the amount of useful information contained in their respective sequences. The phylogenetic trees constructed from each gene were then weighted against the composite sequence dataset to identify the marker that best reproduced the information resulting from the polygenic tree.

Materials and methods

Sequences. 16S rDNA, *amoA*, and *amoB* partial gene sequences from a total of 20 AOB strains of the β- and γ-subclasses of Proteobacteria were obtained from the databases and used in this study (Table 1). The sequences of two methane oxidizers, *Methylocystis* sp. and *Methylosinus trichosporium*, from the β- and γ-subclass of Proteobacteria, respectively, were also included and used as outgroups for phylogenetic reconstruction.

Mutational model. Multiple sequence alignments were performed with CLUSTAL W [51] and refined manually. The proportions of variable and conserved positions were calculated with DNAsp v4 [43]. Silent and effective mutations in the protein-encoding genes *amoA* and *amoB* were manually checked by comparing the DNA sequences with the translated amino-acid sequences.

The hypothesis of neutrality in nucleotide substitution was tested using Tajima's D test [50], included in the software MEGA v.2.1 [26]. The test was independently performed for every marker and for the three positions of the codons from the *amoA* and *amoB* sequences. Substitution saturation was

Table 1. Source of the sequences used in this study

	16S rDNA		*amoA*		*amoB*	
	Accession number	Reference	Accession number	Reference	Accession number	Reference
Nitrosomonas europaea Nm50	M96399	[17]	AJ298710	[1]	AJ555508	[6]
Nm. aestuarii Nm36	AJ298734	[2]	AJ298707	[1]	AJ555504	[6]
Nm. eutropha Nm57	AY123795	[37]	AJ298713	[1]	AJ555506	[6]
Nm. europaea L08050	AB070982	[45]	L08050	[30]	L08050	[30]
Nm. sp. K794	AB031960	Yokoyama et al., unpublished	AB031869	Yokoyama et al., unpublished	AB031869	Yokoyama et al., unpublished
Nitrospira sp. NpAV – copy1	Y10127	[28]	AF032438	[23]	AF032438	[32]
Ns. sp. NpAV – copy2	Y10127	[28]	AF016003	[23]	AF016003	[23]
Ns. sp. Nsp2	AY123802	[38]	AY123822	[38]	AJ555494	[6]
Ns. sp. 40KI	X84656	[52]	AJ298687	[1]	AJ555496	[6]
Ns. sp. Ka4	AJ012106	Aakra et al., unpublished	AJ298697	[1]	AJ555497	[6]
Ns. sp. B6	X84657	[52]	AJ298690	[1]	AJ555498	[6]
Ns. sp. Nv6	AY123805	[38]	AY123826	[38]	AJ555499	[6]
Ns. sp. Nsp1	AY123808	[38]	AY123828	[38]	AJ555500	[6]
Ns. multiformis Nl13	AY123807	[38]	AJ298702	[1]	AJ555501	[6]
Ns. sp. AF	X84658	[52]	AJ298689	[1]	AJ555502	[6]
Ns. sp. Nsp17	AY123804	[38]	AY123825	[38]	AJ555503	[6]
Ns. sp. AHB1	X90820	[40]	X90821	[40]	X90821	[40]
Nitrosococcus sp. AF153344	AF153343	[32]	AF153344	[32]	AF153344	[32]
Nc. oceani C-107	M96395	[17]	AF047705	[32]	AF047705	[32]
Nc. halophilus Nc4	AF287298	[37]	AF272521	[37]	AJ555509	[6]
Methylocystis sp. M	U81595	[29]	U81596	[29]	U81596	[29]
M. trichosporium OB3b	Y18947	[8]	U31650	[16]	U31650	[16]

determined with the index developed by Xia et al. [58] which is included in the DAMBE software [57]. This test is based on the notion of entropy in information theory and yields a critical value permitting the saturation degree of a given set of aligned sequences to be assessed. The same saturation index was calculated for the first, second, and third codon positions in *amoA* and *amoB*. In addition, the entire sequences of the three markers were tested individually for saturation.

The nucleotide substitution model best fitting the variations observed in the 16S rDNA, *amoA*, and *amoB* partial sequences was determined using the software MODELTEST 3.04 [36]. This program allows the most appropriate among 56 models of nucleotide substitution to be chosen.

Phylogenetic analysis. Neighbor-joining (NJ) trees for 16S rDNA, *amoA*, and *amoB* genes were generated from the corresponding matrix of nucleotide divergence between sequences using the program MEGA2 [26]. Maximum parsimony (MP) trees were also constructed for each marker using the software PAUP 4.0b [49]. To reduce the computational time required by the parsimony algorithm when carried out with a heuristic search, a TBR branch-swapping value of 100 was used. Confidence in the branching points was obtained with 1000 bootstrap replicates. The incongruence length difference test (ILD) [10] was conducted as implemented in the PAUP 4.0b software package (partition homogeneity test) and used to determine whether 16S rDNA, *amoA*, and *amoB* sequence datasets provided similar phylogenetic information. The overall NJ and MP trees including the polygenic composite sequences were constructed applying the same variables used for the construction of individual gene trees.

Tree topologies were compared using maximum likelihood, minimum evolution, and parsimony criteria. First, the topologies were analyzed according to the modified Kishino and Hasegawa test [44], computing the log-likelihoods per site for each tree and comparing the total log-likelihoods among topologies [11]. Minimum evolution (ME) scores were then compared for each topology. Finally, the number of steps and both the consistency and retention indices of the parsimony analysis for each tree were computed.

Results

Quantitative aspects of gene variation. Sequences of *amoA* contained the highest proportion of polymorphic sites. Of the 399 sites in this gene, 203 (62.15%) were found to be variable; of these, 11.27% were silent and 50.88% effective. In *amoB*, 180 (45.80%) out of 393 genes were variable and all of them were effective. Of the 1014 16S rDNA genes analyzed, 278 (27.42%) were variable. There were 231 parsimony-informative sites in 16S rDNA, 232 in *amoA*, and 172 in *amoB*. Approximately 50% of the nucleotide substitutions in *amoA* affected the third position of the codon (Table 2), while in the case of *amoB* the variable positions were evenly distributed. Furthermore, 44.27% of the polymorphisms detected in the third base-pair of *amoB* were silent substitutions, i.e., they had no effect on the amino-acid sequence.

Table 2. Variable positions in *amoA* (n = 133) and *amoB* (n = 131) for each codon position

	1st position	2nd position	3rd position	
	Number (%)	Number (%)	Number	
			Silent (%)	Effective (%)
amoA	72 (54.14)	51 (38.35)	125 (93.98)	0 (0.00)
amoB	61 (46.56)	57 (43.51)	58 (44.27)	3 (2.29)

Neutrality and substitution saturation. The dataset fit the model of neutral molecular evolution. In fact, the results of Tajima's D test indicated no significant skew in the entire sequences of the three markers in the case of *amoB* (0.505, 0.421, and 0.623 for the positions 1, 2, and 3, respectively). However, this test revealed a significant excess of polymorphisms in the third position of the codons in *amoA* (3.131 in contrast with the values 1.091 and 0.315 for the positions 1 and 2, respectively). These results agreed with measurements of substitution saturation, which produced a strong signal in the third position of *amoA* codons (Fig. 1). The persistent accumulation of changes in these specific sites in *amoA* may produce a loss of phylogenetic information. No substitution saturation was detected in the other two markers.

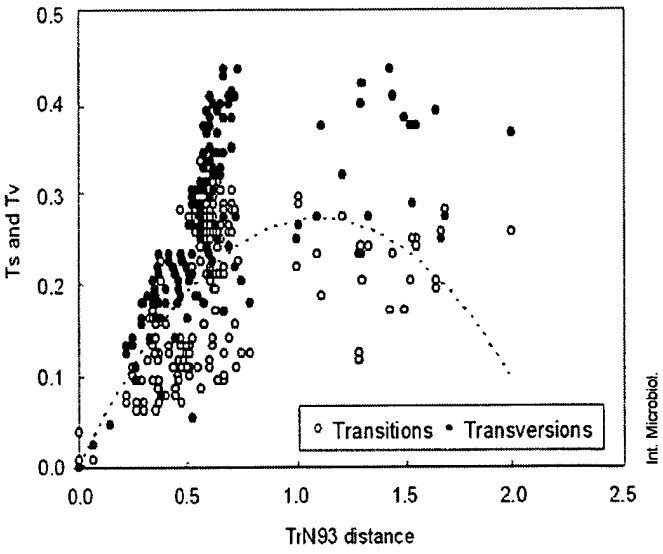

Fig. 1. Saturation diagram for the third position of the codon in *amoA*, showing the rate of transitions and transversions versus the Tamura-Nei 1993 distance.

Phylogenetic and topology analyses. The evolution of each gene can be described by a distinct substitution model (Table 3). Tamura-Nei 1993 (TrN93) is the nucleotide substitution model including the greatest number of parameters, and the one best fitting the combined dataset. The models Hasegawa-Kishino-Yano 1985 (HKY) and Felsenstein 1981 (F81), obtained for *amoA* and *amoB*, respectively, can be considered simplifications of TrN93. TrN93 was applied to all trees based on genetic distances, with a single transition

Table 3. Nucleotide substitution models, obtained base frequencies, and γ-shape distribution values for 16S rDNA, *amoA*, and *amoB* genes, and for the polygenic dataset [36]. Abbreviations: TrN, Tamura Nei 1993; HKY, Hasegawa-Kishino-Yano 1985; F81, Felsenstein 1981; I, proportion of invariable sites; G, γ-shape parameter

		16S rDNA	*amoA*	*amoB*	Polygenic
Model selected		TrN + I + G	HKY + G	F81 + G	TrN + I + G
Base frequencies	A	0.2668	0.1820	0.2321	0.2258
	C	0.2074	0.2990	0.2502	0.2491
	G	0.3108	0.2546	0.3148	0.3026
	T	0.2150	0.2644	0.2029	0.2225
γ-Shape distribution values		0.5645	0.3546	0.1026	0.3824

type and a single substitution rate when the selected models were HKY and F81, respectively.

For each marker, an NJ tree was constructed using the appropriate nucleotide substitution model (Table 3, Fig. 2). The trees constructed by MP showed topologies similar to those of their NJ counterparts (data not shown). In all cases, the *Nitrosomonas* and *Nitrosospira* radiations grouped together, and the γ-proteobacterial nitrosococci branched separately. This agrees with the classical phylogenetic topology of AOB. Likewise, two different clusters were distinguishable within the β-subgroup of ammonia oxidizers, as *Nitrosomonas* and *Nitrosospira* clearly formed two separate clades. Nevertheless, the allocation of *Nitrosomonas aestuarii* Nm36 was uncertain, since it grouped within the *Nitrosospira* cluster when using *amoB* as a molecular marker but fell within the *Nitrosomonas* group when using 16S rDNA or *amoA*. The ILD test corroborated ($P < 0.001$) the incongruence between the phylogenetic information provided by the three markers. However, since under some circumstances combining sequences with different phylogenetic histories can improve the accuracy of phylogenetic analysis [56], polygenic trees were constructed.

The consensus polygenic trees generated by MP and NJ are presented in Fig. 3. The topologies of the two trees were similar and consistent with both the standard classification of AOB and the results previously obtained with each of the three markers. In this polygenic analysis, *Nm. aestuarii* Nm36 was considered to be the most divergent *Nitrosomonas*.

All topological evaluations (likelihood, minimum evolution, and parsimony criteria) indicated that the MP tree obtained from the composite dataset displayed the most probable topology (Table 4). Similar values were obtained for the rest of the trees, which indicated that they were not significantly worse than the best-supported tree.

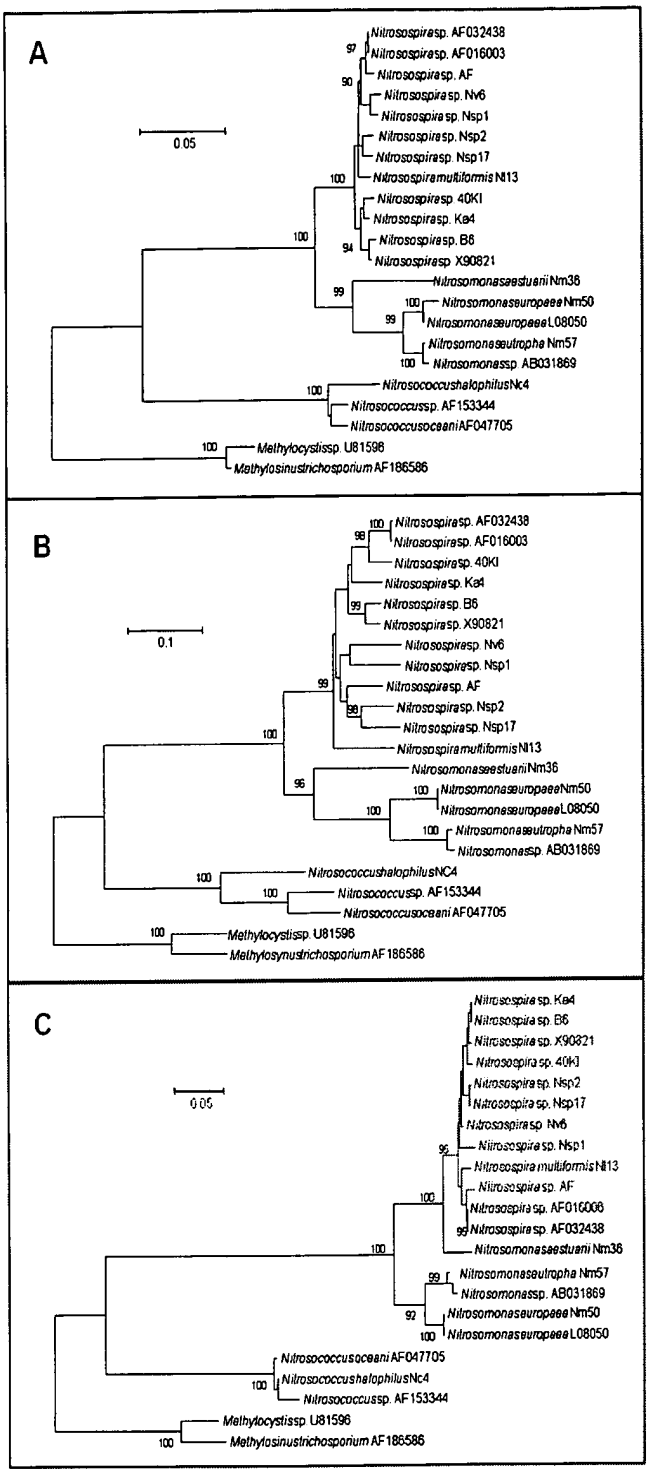

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Fig. 2. Neighbor-joining (NJ) tree from alignments of (**A**) 16S rDNA, (**B**) *amoA*, and (**C**) *amoB* sequences. The model of nucleotide substitution used in every case is specified in Table 2. Bootstrap values above 75% are shown; the scale bar represents the number of estimated changes per nucleotide. 16S rDNA, *amoA*, and *amoB* genes sequences from *Methylocystis* sp. U81596 and *M. trichosporium* AF047705 were used as outgroups in (**A**), (**B**), and (**C**).

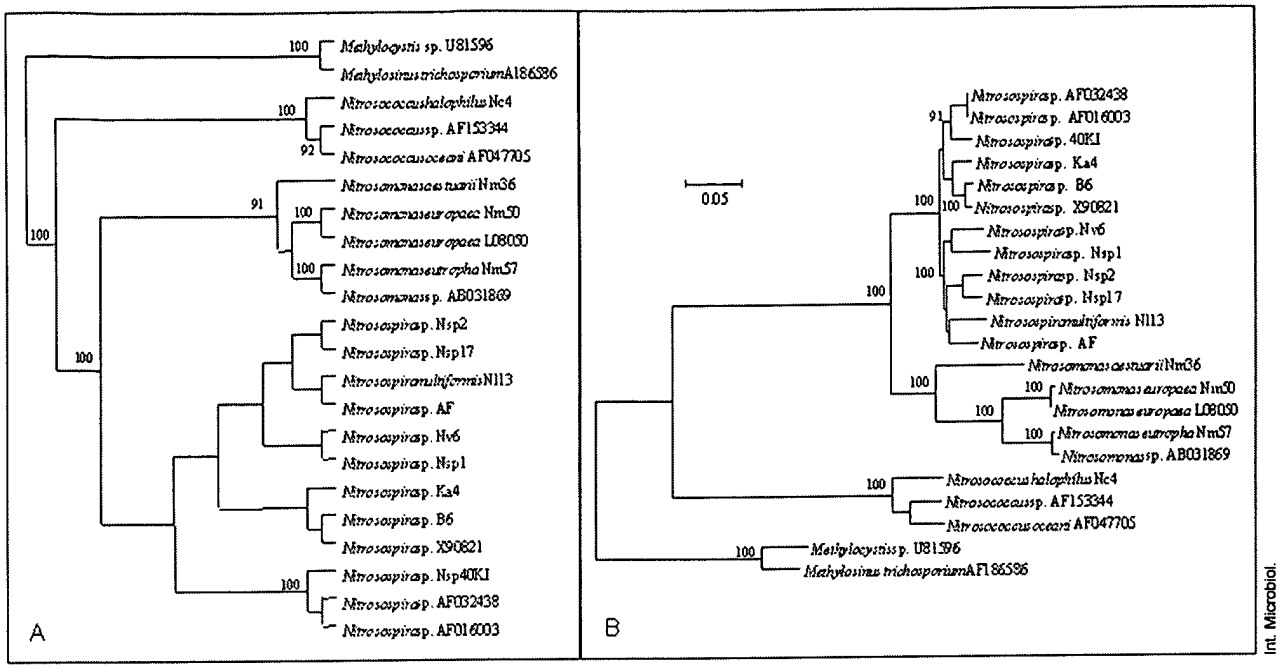

Fig. 3. Maximum parsimony (MP) consensus tree (**A**), and neighbor-joining (NJ) distance tree (**B**) generated from an alignment of partial *amoA*, *amoB*, and 16S rDNA sequences from ammonia-oxidizing bacteria (AOB). The distance matrix for the NJ tree was constructed based on the Tamura-Nei 1993 substitution rate. Bootstrap values (in percentage) are indicated. Partial 16S rDNA, *pmoA*, and *pmoB* gene sequences from *Methylocystis* sp. U81596 and *M. trichosporium* AF047705 were used as outgroups.

Discussion

Correct classification of any bacterial group requires the input of different genetic and phenetic characters, which is not possible when using uncultured bacteria from natural environments [39]. Alternatively, a polygenic approach leads to more accurate estimations of the diversity and composition of natural populations [34,35], although care must be taken when combining datasets from different markers [9,56]. Gene *amoA* encodes the active site of ammonia monooxygenase [20], which makes it difficult for effective mutations to occur in this gene. In *amoA*, 50% of the mutations were detected in the third base-pair (see Table 2), which showed a large accumulation of nucleotide changes at this position. As a consequence, the third position of the codon in *amoA* is strongly saturated (see Fig. 1) and deviates from neutrality, suggesting that this position has experienced selective pressures different from those of the other two positions [24]. The persistent accumulation of changes in these specific sites in *amoA* may produce a loss of phylogenetic information. By contrast, in the case of *amoB*, ca. 55% of the conserved sites were detected in all codon positions (Table 2). Moreover, ca. 70% of the amino-acid variations observed in the deduced partial amino acid sequences of AmoB proteins are conservative (data not shown).

The general topologies of the constructed trees were almost identical, with one exception. Although taxonomically

Table 4. Comparisons among tree topologies. KHT, Shimodaira and Hasegawa (1999) likelihood analysis (likelihood parameters used are described in Table 3); ME, minimum evolution scores reported by PAUP 4.0b [49]; parsimony length, CI and RI, respectively, the number of steps, the consistency index, and the retention index of the parsimony analysis for each topology as obtained using PAUP 4.0b

	Polygenic NJ + TN93	Polygenic PARS	*amoA* NJ + TN93	*amoA* PARS	*amoB* NJ + TN93	*amoB* PARS	16S rDNA NJ + TN93	16S rDNA PARS
KHT	−12764.5	−12755.1*	−12783.0	−12786.5	−12996.1	−13039.6	−12884.7	−12872.7
ME	1.67865	1.67904*	1.69236	1.75645	1.79980	1.79070	1.69853	1.71277
Parsimony length	2170	2168*	2176	2184	2260	2264	2206	2201
Parsimony CI	0.633	0.633*	0.631	0.629	0.608	0.606	0.622	0.624
Parsimony RI	0.771	0.772*	0.770	0.767	0.746	0.744	0.761	0.762

* Statistically significant best tree.

classified into the genus *Nitrosomonas*, the strain *Nm. aestuarii* Nm36 showed significant phylogenetic distances, supported by high bootstrap values, from the central cluster of *Nitrosomonas* when using 16S rDNA and *amoA* gene sequences. By contrast, *Nm. aestuarii* Nm36 grouped together with the *Nitrosospira* lineage when using *amoB*, but a considerable phylogenetic distance also distinguished this strain from the rest of the nitrosospiras. In the polygenic tree, *Nm aestuarii* Nm36 again grouped with the *Nitrosomonas* cluster. Purkhold et al. also reported the ambiguous phylogenetic arrangement of this species depending on the treeing method employed and the type of sequences used [37]. Therefore, this strain should be further studied in order to clarify its phylogenetic affiliation. Moreover, it would be of interest to determine whether *amoB* of *Nm. aestuarii* Nm36 has followed a different pattern of evolution and represents the ancestral state within the *Nitrosomonas* cluster, or whether it is a case of lateral gene transfer.

The composite dataset, consisting of 16S rDNA, *amoA*, and *amoB* sequences, provided more information than any of the three markers alone, and therefore resulted in the most accurate classification. Thus, the marker leading to the tree best-fitting the information of the entire dataset should be the one chosen for taxonomic and diversity studies. As expected, results of a comparison between all of the trees and the data obtained using the likelihood, minimum evolution, and parsimony criteria showed that the polygenic MP tree was the best. However, phylogeny could be inferred using any of the markers. Although our results may be biased due to both the sequence sizes of the markers (16S rDNA: 1014 bp; *amoA*: 399 bp; *amoB*: 393 bp) and the number of parsimonic informative sites, they support 16S rDNA as a good phylogenetic marker, especially concerning the avoidance of ambiguous classifications. Several authors have recently reaffirmed the potential of 16S rDNA sequences for drawing phylogenetic inferences [3,27,38]. Nonetheless, obtaining the 16S rDNA gene from environmental samples is time-consuming and tedious. It requires the cloning of all 16S rDNA genes present in the sample and then distinguishing the 16S rDNA genes belonging to AOB from the rest.

By contrast, environmental population studies based on the analysis of *amoA* or *amoB* present some significant advantages: the genes are AOB-specific, are large enough to allow quick fingerprinting of natural communities, and provide a phylogeny consistent with the current taxonomic outlines. Nevertheless, Oved et al. [33] and Nicolaisen and Ramsing [31] reported the amplification of non-AOB sequences when using *amoA* sequences in a PCR–denaturing gradient gel electrophoresis (DGGE) approach. Our experiments based on *amoB* amplification combined with DGGE resulted in the establishment of a sensitive and reliable screening method to detect and identify AOB in environmental samples (data not shown). Additionally, the benefit of using *amoB* in ecophysiology studies is the ability to distinguish methane-oxidizing bacteria from AOB on a simple agarose gel [6].

Based on the results reported here, for taxonomic purposes we strongly recommend sequencing16S rDNA, *amoA* and *amoB* genes, and to construct a polygenic tree. Since the third position of the codon in *amoA* is saturated, and due to the non-AOB sequences retrieved by other authors when using this gene [31,33], the use of *amoB* is recommended when carrying out environmental ecophysiology studies. *amoB* allows fingerprinting techniques, such as terminal restriction fragment length polymorphism (tRFLP) and DGGE, to be performed, and results in a reliable phylogenetic profile. Moreover, when using *amoB* as a marker, the methane-oxidizers present in the sample can be quickly and easily distinguished from AOB, which may be of great help in analyzing complex samples.

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Análisis poligénico de cepas de bacterias oxidadoras de amoníaco por medio de los genes 16S rDNA, *amoA* y *amoB*

Resumen. Encontrar un marcador molecular único capaz de proporcionar rápidamente información filogenética rigurosa y útil facilitaría evaluación de la diversidad de las bacterias oxidadoras de amoníaco en muestras ambientales. En esta clase de estudios no se puede utilizar simultáneamente más que uno de los marcadores disponibles. Los genes 16S rDNA, *amoA* y *amoB* se evaluaron individualmente para identificar el que se ajusta mejor a la información proporcionada por el conjunto de datos de los tres genes. Se compararon los árboles de Neighbor-Joining, basados en las distancias, y los árboles de máxima parsimonia basados en las secuencias conocidas de los tres genes mencionados, y se analizaron en relación con los árboles poligénicos construidos con la información combinada proporcionada por los tres genes. Los árboles de máxima parsimonia resultaron más fieles que los basados en las distancias, y la topología poligénica era la que mejor se ajustaba a la información contenida en las secuencias. Sin embargo, la información taxonómica y filogenética proporcionada por los tres marcadores por separado también resultó válida. Por tanto, cualquiera de los dos marcadores funcionales (*amoA* o *amoB*) se puede utilizar para detectar los oxidantes del amoníaco en estudios ambientales en los que solamente puede usarse un gen. [**Int Microbiol** 2005; 8(2):103-110]

Palabras clave: bacterias oxidadoras de amoníaco · 16S rDNA · *amoA* · *amoB* · análisis poligénico

Análise poligénico de cepas de bactérias oxidadoras de amoníaco através dos genes 16S rDNA, *amoA* e *amoB*

Resumo. Encontrar um marcador molecular único capaz de proporcionar rapidamente informação filogenética rigorosa e útil facilitaria avaliação da diversidade das bactérias oxidadoras de amoníaco em amostras ambientais. Nesta classe de estudos não é possível utilizar simultaneamente mais que um dos marcadores disponíveis. Os genes 16S rDNA, *amoA* e *amoB* foram avaliadas individualmente para identificar o que se ajusta melhor à informação proporcionada pelo conjunto de dados dos três genes. Foram comparadas as árvores filogenéticas de Neighbor-Joining, baseadas nas distâncias, e as árvores de máxima parcimônia baseadas nas seqüências conhecidas dos três genes mencionados, e foram analisadas em relação com as árvores poligénicas construídas com a informação combinada proporcionada pelos três genes. As árvores de máxima parcimônia resultaram mais fiéis que as baseadas nas distâncias, e a topologia poligénica foi a que melhor se ajustou à informação contida nas seqüências. No entanto, a informação taxonômica e filogenética proporcionada pelos três marcadores separadamente também resultou válida. Portanto, qualquer dos dois marcadores funcionais (*amoA* ou *amoB*) pode-se utilizar para detectar os oxidantes do amoníaco em estudos ambientais nos quais somente pode-se usar um gene. [**Int Microbiol** 2005; 8(2):103-110]

Palavras chave: bacterias oxidadoras de amoníaco · 16S rDNA · *amoA* · *amoB* · análise poligénico